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(57) Abstract

Vector construct providing for inducible overproduction of poly-β-hydroxyalkanoate ("PHA"), including poly-β-hydroxybutyrate (PHB), in prokaryotes, including *Escherichia coli* in particular. The vector constructs provide for one or more of (a) regulated transcription of the *phb* operon, thereby providing numerous copies of mRNA suitable for production of PHA, (b) multiple copies of the vector construct upon heat induction, thereby providing numerous copies of the *phb* operon for production of PHA and (c) an altered *phb* operon wherein the *phbC* Shine-Dalgarno sequence, or ribosome binding site, is replaced with a consensus Shine-Dalgarno sequence, such as the *lac* Shine-Dalgarno sequence. Further, the vector constructs may include a stabilization locus. Also, methods of producing PHA from such high production vector constructs, including methods wherein PHA is produced without addition of a chemical inducer such as IPTG or an antibiotic, bacterial host cells transformed with such vector constructs, and PHA produced according to the methods of the present invention.

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Description

IMPROVED PRODUCTION OF POLY-BETA-HYDROXYALKANOATES IN TRANSFORMED PROKARYOTIC HOSTS

Cross-Reference to Related Applications

This application is a continuation-in-part of pending application U.S. Serial No. 08/035,433, filed March 24, 1993, which is a continuation-in-part of pending application U.S. Serial No. 07/890,925, filed May, 29 1992 and a continuation-in-part of pending application U.S. Serial No. 07/767,008, filed September, 27, 1991, all of which are incorporated herein by reference.

Technical Field

The present invention relates generally to the production of carbon storage polymers, and more specifically to the production of poly-β-hydroxyalkanoates such as poly-β-hydroxybutyrate.

Background of the Invention

Poly-β-hydroxyalkanoates (PHAs) are a heterogeneous family of biodegradable aliphatic polyesters that are typically formed of monomers from 4-10 carbons. PHAs can be, for example, "random" copolymers wherein the copolymer comprises poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV) dispersed randomly in the polymer backbone, or "semi-random," or blocked, copolymers wherein the copolymer comprises long or short chains of one particular PHA, for example PHB, that is separated by long or short chains of other PHAs, for example, randomly dispersed PHB and PHV.

PHAs are produced under certain conditions, such as where a culture is first incubated on a 3-carbon nutrition source until the culture reaches late log phase growth, then the culture is incubated on a second carbon source such as glucose, fructose or sucrose.

PHAs, including PHB, are synthesized by the action of three enzymes: β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase (Oeding and Schlegel, *Biochem. J. 134*:239, 1973; Senior and Dawes, *Biochem. J. 134*:225, 1973). (This reference, and all other references cited herein, are hereby expressly incorporated by reference in their entirety.) β-ketothiolase condenses two acetyl-CoA molecules to acetoacetyl-CoA. Acetoacetyl-CoA reductase reduces this compound to β-hydroxybutyryl-CoA. PHB synthase typically

polymerizes β-hydroxybutyryl-CoA into PHB, although other PHAs are produced under particular conditions, such as those described above.

The PHB biosynthetic pathway of *Alcaligenes eutrophus* H16 has been cloned into *Escherichia coli* (Slater et al., *J. Bact. 170*:4431, 1988; Schubert et al., *J. Bact. 170*:5837, 1988) and the DNA sequence of the pathway has been determined (Peoples and Sinskey, *J. Biol. Chem. 264*:15293, 1989; Peoples and Sinskey, *J. Biol. Chem. 264*:15298, 1989; Janes et al., *Novel Biodegradable Microbial Polymers*, 175, 1990). The operon consists of three genes designated *phbC*, *phbA*, and *phbB*, which encode PHB synthase, β-ketothiolase, and acetoacetyl-CoA reductase, respectively. The transcriptional start site has been determined by deletion analysis (Janes et al., *supra*) and by S1 nuclease mapping (Schubert et al., *J. Bact. 173*:168, 1991). The promoter for the *phb* operon is very similar to the *E. coli* promoter recognized by σ70 (Rosenberg and Court, *Ann. Rev. Genet. 13*:319, 1979), and the *phb* operon is transcribed in *E. coli*. The transcriptional start site is located approximately 300 bp upstream from the start of the *phbC* translational start site.

Clones that carry the *phb* operon on a multicopy plasmid can produce PHAs to levels as high as 80% of cell weight in media containing whey, a waste product of cheese production that contains a high concentration of lactose (Janes et al., *supra*). The use of whey, and other low-cost food sources such as sucrose-containing food sources, has been a major stride in decreasing the overall cost of industrial scale production of PHAs. Another significant expense in the industrial production of PHAs is the inclusion of an antibiotic in the growth culture of bacteria useful for PHA production. For example, in a 100,000 liter fermentor, the cost of producing about 22,000 pounds of PHB (which would be about $22,000 if no antibiotic were necessary) is increased by over $14,000 when the commonly used antibiotic chloramphenicol is added to the mixture.

Further information with respect to the production of PHB and PHAs, including PHB, can be found the following United States patent applications: U.S. Serial No. 07/362,514, filed June 7, 1989; U.S. Serial No. 07/528,549, filed May 25, 1990; U.S. Serial No. 07/705,806, filed May 24, 1991; U.S. Serial No. 07/767,008, filed September, 27, 1991; U.S. Serial No. 07/890,925, filed May 29, 1992; and, U.S. Serial No. 08/035,433 filed March 24, 1993. As noted above, each of these references is expressly incorporated herein in its entirety.

One desirable advantage in the production of PHAs and/or PHB would be the regulation of transcription and/or translation of the *phb* operon. Another desirable advantage would be the provision of bacterial strains able to produce PHAs without the inclusion of an antibiotic in the growth medium. These and other advantages provided by the present invention will become apparent in the following discussion.

Summary of the Invention

The present invention provides nucleic acid vector constructs capable of regulating the transcription and/or translation of a *phb* operon. The present invention also provides methods of using such constructs to produce PHAs, host cells transformed with such constructs, and PHA and PHB produced according to the methods of the present invention.

Accordingly, in one aspect, the present invention provides a nucleic acid vector construct comprising a) a promoter comprising a -35 region of a *trp* promoter operably linked to a -10 region of a *lac* promoter, and b) an operator region of the *lac* promoter, said promoter being operably linked to said operator region and to a *phb* operon. Preferably, the promoter is a *tac* promoter, and the *phb* operon is derived from *Alcaligenes eutrophus*. Further preferably, the vector constructs comprise a consensus Shine-Dalgarno sequence operably linked to a *phbC* gene of the *phb* operon. Alternatively, the consensus Shine-Dalgarno sequence is added to a *phb* operon that is otherwise not subject to overproduction of PHA. Still further preferably, the consensus Shine-Dalgarno sequence is a *lac* Shine-Dalgarno sequence. The consensus Shine-Dalgarno sequence may replace a native *phbC* Shine-Dalgarno sequence, or the consensus Shine-Dalgarno sequence may be in addition to the native *phbC* Shine-Dalgarno sequence. Also preferably, the vector constructs further comprise a stabilization locus such as *parB*.

In further embodiments, the present invention provides vector construct having all of the essential characteristics of one, or more, of plasmids pJM9227, pJM9229, pJM9230, pJM9231, pJM9232, pJM9233, pJM9234, pJM9235, pJM9236, pJM9237, pJM9238, pJM9375, pJM9376, pJM9117 and pJM9131. A vector construct having all of the essential characteristics of one of the recited plasmids retains all of the properties described herein for that plasmid. For example, if the recited plasmid has a *trp* -35 region, a *lac* -10 region and a *lac* operator region operably linked to the *phb* operon, and thus the plasmid is capable of being induced by IPTG to overproduce PHAs, then these

properties are maintained. Preferably, the vector construct consists essentially of the nucleic acid sequence of the recited plasmid.

In another preferred embodiment, the vector constructs of the present invention have the *tac* promoter and the *phb* operon separated by a leader having a *cis*-acting positive regulatory element.

In another aspect, the present invention provides a runaway replicon nucleic acid vector construct that includes an expressible *phb* operon. Preferably, such a nucleic acid vector construct has the *phb* operon operably linked to and positioned downstream from a) a promoter comprising a -35 region of a *trp* promoter operably linked to a -10 region of a *lac* promoter, and b) an operator region of the *lac* promoter, the promoter also being operably linked to the operator region. Further preferably, the promoter is a *tac* promoter, the *phb* operon is derived from *Alcaligenes Eutrophus* and/or the runaway replicon vector construct includes a λ pR promoter operably linked to a *repA* gene.

In a further aspect, the present invention provides a method for the production of PHA, comprising: (a) introducing into a prokaryotic host cell a vector construct comprising a) a promoter comprising a -35 region of a *trp* promoter operably linked to a -10 region of a *lac* promoter, and b) an operator region of the *lac* promoter, the promoter being operably linked to the operator region and to a *phb* operon; (b) culturing the host cell in an appropriate medium; (c) adding an inducer to the medium, the inducer being capable of activating the promoter; and (d) further culturing the host cell for a time sufficient to produce PHA.

In preferred embodiments, the inducer is IPTG and the promoter is a *tac* promoter, and the method, in step (a), further comprises introducing a *lacIq* gene into the prokaryotic host cell.

In yet another aspect, the present invention provides a method for the production of PHA, comprising: (a) introducing into a prokaryotic host cell a runaway replicon vector construct comprising an expressible *phb* operon and a λ pR promoter operably linked to a *repA* gene, and a λcI857 gene; (b) culturing the host cell in an appropriate medium; (c) increasing the temperature of the host cell, thereby inducing the runaway replicon vector construct; and (d) further culturing host cell for a time sufficient to produce PHA.

In a preferred embodiment, the method further comprises, during step (b), determining whether the culture of the host cells is growing rapidly or slowly; and during step (c), increasing the temperature early in a log phase of a growth cycle of the culture when the culture is fast-growing, or increasing the

temperature late in a log phase of a growth cycle of the culture when the culture is slow-growing. In another preferred embodiment, the increase in temperature is to at least 33°C when the vectors are maintained in *Klebsiella* and at least 36°C when the vectors are maintained in *E. coli*.

In a preferred embodiment, these aspects of the invention further comprise the step of isolating PHA, including PHB, from the cultured host cell. In another preferred embodiment, the host cell is an Enterobacteriaceae host cell, further preferably an *E. coli, Klebsiella* or *Klebsiella aerogenes*. Still further, the culture medium preferably does not include an antibiotic.

In yet another preferred embodiment, the runaway replicon vector construct further includes a *tac* promoter, or other promoter inducible by a chemical inducer, such as IPTG, as described herein, and the method further comprises obtaining such overproduction of PHAs without the addition of the chemical inducer (such as IPTG).

In yet a further aspect, the present invention provides an Enterobacteriaceae host cell containing one, or more, vector constructs as described above. The host cell is preferably an *E. coli* or *Klebsiella*.

In still another aspect, the present invention provides PHA and/or PHB produced according to the methods described herein.

These and other aspects of the present invention will become evident upon reference to the following detailed description, examples and attached drawings.

Brief Description of the Drawings

Figure 1 depicts the nucleotide sequence of a series of vector constructs having certain transcriptional and/or translational fusions to the *phb* operon. The nucleotide sequence denoted "a" comprises a *phb* promoter operably linked to a putative *phbC* Shine-Dalgarno sequence (denoted "SD" in the figure) (Seq. ID No. _____). This nucleotide sequence is present in the plasmids pJM9131 and pJM9117. The nucleotide sequence denoted "b" comprises a *tac* promoter and a *phbC* Shine-Dalgarno sequence wherein there is an approximately 72 base pair leader sequence prior to the structural gene (Seq. ID No. _____). This nucleotide sequence is present in the pJM9229 and pJM9236 vector constructs. The nucleotide sequence denoted "c" comprises a *tac* promoter and a putative *phbC* Shine-Dalgarno sequence wherein there is an approximately 355 base pair leader prior to the structural gene (Seq. ID No. _____). This nucleotide sequence is present in the pJM9232 and pJM9238

vector constructs. The nucleotide sequence denoted "d" comprises a *tac* promoter and two *lac* Shine-Dalgarno sequences (Seq. ID No. _____). This nucleotide sequence is present in the pJM9375 vector construct. The nucleotide sequence denoted "e" comprises a *tac* promoter, two *lac* Shine-Dalgarno sequences and a *phbC* Shine-Dalgarno sequence (Seq. ID No. _____). This nucleotide sequence is present in the pJM9376 vector construct.

Figure 2 depicts a map of the vector construct pJM8801 (formerly known as p4A), which contains the *phb* operon, and the construction of the vector construct pJM9002, which is an 8.10 kb plasmid produced by cleaving the *Eco* RI/*Hind* III *phb* operon-containing fragment from p4A and ligating the fragment into the same sites of pBluescript SK+. Figure 2 also depicts the construction of the pJM9131 vector construct, which is an 8.55 kb plasmid derived from pJM8801, but a kanamycin gene block is located at the *Eco* RI site, and ampicillin resistance has been removed by *Dra* I digestion. Figure 2 further depicts the construction of the pJM9226 vector construct, which is a 3.10 kb plasmid comprising the *tac* promoter and a *lac* Shine-Dalgarno sequence ligated into the 3 kb *Hind* III-*Bam* HI fragment of pJM9002.

Figure 3 depicts a map of the vector construct pJM8703, which is an 8.50 kb plasmid comprising the *phb* operon cloned into pTZ18U from United States Biochemicals. The transcription pathway starts at the *Kpn* I site and ends at the *Eco* RI site. Figure 3 also depicts the construction of the pTZ18U-4c vector construct, which is a 7.00 kb plasmid produced by digesting pJM8703 with *Sph* I and *Bam* HI, then deleting from the *Bam* HI to base 835 of the published sequence followed by religation. Figure 3 further depicts the construction of the pSP72/PHB vector construct, which is a 8.50 kb plasmid comprising the *Eco* RI/*Pst* I fragment from pJM8703 ligated into the same sites of pSP72 (Promega). Figure 3 also depicts the construction of the pJM8905 vector construct, which is an 8.49 kb plasmid comprising a *Pst* I partial of *Eco* RI-digested pJM8703 and ligated into pSP72, which was in turn digested with *Xho* I and *Eco* RI to provide a *phb* operon-containing fragment that was ligated into pGEM7Zf+. Figure 3 further depicts the construction of the pJM9117 vector construct, which is a 10.13 kb plasmid that contains the *phb* operon from pGEM7-PHBr cloned into the *Bam* H I site of pRA89, which is a 5.13 kb plasmid that is inducible above 41°, and typically has a basal copy number of 1. The pJM9117 vector construct was formerly known as pRA89/PHB/Fo.

Figure 4 depicts the construction of the pJM9227 vector construct, which is a 7.20 kb plasmid comprising the *Bst* BI-*Bam* HI fragment from

pJM8905 inserted into the *Bam* H1 site of pJM9226 to provide a vector construct having a *tac::phb* fusion. Figure 4 also depicts construction of the pJM9228 vector construct, which is an 8.50 kb plasmid comprised of a kanamycin gene block inserted into the *Eco* RI site downstream of the *phb* operon of pJM9227 (*i.e.*, pJM9228 is similar to pJM9227 with kanamycin resistance). Figure 4 further depicts the construction of the pJM9229 vector construct, which is a 7.80 kb plasmid comprising a 0.71 kb deletion in the *bla* gene of pJM9228 (*i.e.*, the ampicillin resistance of pJM9228 was deleted).

Figure 5 depicts the construction of the pJM9230 vector construct, which is a 7.50 kb plasmid that includes the *phb* operon-containing *Hind* III-*Eco* RI fragment from pTZ18U-4c ligated into the *Bam* HI site of pJM9226. Figure 5 also depicts the construction of the pJM9231 vector construct, which comprises an 8.80 kb plasmid including a kanamycin gene block inserted into the *Spe* I site downstream of the *phb* operon in pJM9230 (*i.e.*, pJM9231 is similar to pJM9230 with kanamycin resistance). Figure 5 also depicts the construction of the pJM9232 vector construct, which is an 8.10 kb plasmid, including a 0.71 kb deletion in the *bla* gene of pJM9231 (*i.e.*, the ampicillin resistance of pJM9231 was deleted).

Figure 6 depicts the construction of the pJM9233 vector construct, which is a 9.10 kb plasmid including the *Hind* III-*Eco* RI fragment from pJM9227 (which contains the *tac::phb* fusion), ligated into the filled-in *Bam* HI site of pRA89, and the construction of the pJM9234 vector construct, which is a 9.10 kb plasmid similar to the pJM9233 vector construct, except that the *phb* operon is in the reverse orientation.

Figure 7 depicts the construction of the pJM9235 vector construct, which is a 9.40 kb plasmid that is similar to the pJM9233 vector construct, except that the insert was placed in the pRA90 vector construct, which is a 5.37 kb plasmid that is inducible at 41°, has a basal copy number of 1 and is resistant to chloramphenicol at 30 μg/ml. Figure 7 also depicts the construction of the pJM9236 vector construct, which is a 9.40 kb plasmid that is similar to the pJM9235 vector construct, except that the *phb* operon is in the reverse orientation.

Figure 8 depicts the construction of the pJM9237 vector construct, which is a 9.70 kb plasmid comprising the *Hind* III-*Spe* I fragment from pJM9230 vector construct (which contains the *tac*-leader-*phb* operon fusion) ligated into the filled-in *Bam* HI site of pRA90, and the pJM9238 vector

construct, which is a 9.70 kb plasmid similar to the pJM9237 vector construct except that the *phb* operon is in the reverse orientation.

Figure 9 depicts the pMS421 vector construct, which is a 5.5 kb plasmid containing the *lac* I gene.

Figure 10 depicts a series of graphs indicating the synthesis of *phb* operon gene products in the *E. coli* strains HMS174 pJM9131 (panel a), HMS174 pJM9232 pMS421 (panel b), HMS174 pJM9117 (panel c), and HMS174 pJM9238 (panel d), as a function of percentage of total protein over time.

Figure 11 depicts the construction of the pJM9275 and pJM9376 vector constructs, which are 7.20 kb plasmids constructed by *Exo* III deletion from the *Bst* BI site in pJM9230 to potentially remove all or a part of the native *phb* Shine-Dalgarno sequence, while inserting a consensus Shine-Dalgarno sequence.

Figure 12 depicts a series of graphs indicating the production of PHB in the *E. coli* strains HMS174 pJM9238 (panel a), HMS174 pJM9117 (panel b), HMS174 pJM9231 pMS421 (panel c), and HMS174 pJM9232 pMS421 (panel d), as a function of time and glucose consumption.

Figure 13 depicts a pair of graphs comparing PHB yield as a percentage of dry weight in clones containing runaway replicon vector constructs.

Figure 14 depicts a pair of graphs comparing plasmid copy number in clones containing a runaway replicon vector construct.

Figure 15 depicts a graph indicating a comparison of PHB production in clones containing a multicopy *tac::phb* vector construct.

Figure 16 depicts a graph indicating PHB production in *E. coli* strain HMS174 pJM9238 at different incubation temperatures.

Figure 17 depicts a graph indicating PHB yield in *E. coli* strain HMS174 pJM9238 as a function of the optical density of a culture at the time of induction.

Figure 18 depicts a graph indicating PHB yield in *E. coli* strain HMS174 pJM9238 with and without chloramphenicol.

Figure 19 depicts a graph indicating a comparison of PHB production in clones containing transcriptional and translational fusions (pJM9375 and pJM9376) versus a vector construct having only a transcriptional modification (pJM9232).

Figure 20 depicts a pair of graphs as follows: Panel a depicts the stability of plasmid pJM9238 *Klebsiella* strain KC2671 over approximately 120 generations when grown in media without chloramphenicol (or other antibiotics). Panel b depicts PHB production in KC2671 pJM9238 at 31°C and 33°C.

Figure 21 depicts a pair of graphs as follows: Panel a depicts the production of PHB in mg/ml in KC2671 pJM9238 over time. The graph also indicates the total dry cell weight of the *Klebsiella* host cells including the PHB. Panel b depicts a comparison of PHB yield in KC2671 for plasmids pJM9131 and pJM9238.

Detailed Description of the Invention

The present invention provides nucleic acid vector constructs suitable for introduction into an appropriate prokaryotic host where the vector constructs provide for regulatable overproduction of PHAs, particularly PHB. The vector constructs are typically plasmids and provide for one or more of (a) regulated transcription of the *phb* operon due to a negatively regulated promoter, preferably comprising a -35 region of a *trp* promoter operably linked to a -10 region of a *lac* promoter, the promoter operably linked (and typically overlapping) an operator region of a *lac* promoter, such promoter being further operably linked to a *phb* operon, thereby providing multiple copies of mRNA suitable for production of PHB, (b) a runaway replicon nucleic acid vector construct that includes an expressible *phb* operon that provides multiple copies of the vector construct upon heat induction, thereby providing numerous copies of the *phb* operon for production of PHB and (c) an altered *phb* operon comprising a consensus Shine-Dalgarno sequence (preferably a *lac* Shine-Dalgarno sequence), operably linked to the *phbC* gene, thereby providing for increased translation of the *phbC* gene; the consensus Shine-Dalgarno sequence may either replace the native *phbC* Shine-Dalgarno sequence or it may be in addition to such native sequence. Representative embodiments of suitable operons and Shine-Dalgarno sequences are depicted in Figure 1 (Seq. ID Nos. _____ to _____). The present invention also provides methods of producing PHA from such high production vector constructs, bacterial host cells transformed with such vector constructs, and PHAs produced according to the methods of the present invention.

Thus, in one aspect, the present invention provides vector constructs that comprise strongly expressed and tightly negatively regulated promoters operably linked to the *phb* operon. Such promoters can be

controllably "turned on" and "turned off" by the introduction of an inducer or a derepressor into its host cell. When "turned on," such promoters permit substantially uninterrupted transcription of a gene (or operon) operably linked thereto (and such promoters are not repressed by substances found in the host cell). When "turned off," such promoters do not permit any substantial transcription of the gene. Methods for determining whether and when such promoters are "on" and "off," as well as the detection of gene products from the linked gene (such as mRNA, proteins or enzymes, or downstream products such as PHAs), are well known in the art, in light of the instant specification.

In a preferred embodiment, the negatively regulated promoter comprises the -35 region of the *trp* promoter operably linked to the -10 region of the *lac* promoter, and the operator region of the *lac* promoter (Russell and Bennett, *Gene 20*:231, 1982). Representative examples of such a promoter are found in Figure 1. The -35 region of a promoter typically comprises an approximately 6- to 12-base sequence centered around the -35 nucleotide (plus or minus two or three nucleotides, measured from the transcription initiation site). Further typically, the -35 region of the *trp* promoter includes the nucleotide sequence TTGACA (Darnell, et al., *Mol. Cell Biol.*, 270-85, 1986) (Seq. ID No. _____). The -10 region, also known as a Pribnow box, typically comprises an about a 6-base sequence centered around the -10 nucleotide (plus or minus two or three nucleotides, also measured from the transcription initiation site). Further typically, the *lac* -10 region includes the nucleotide sequence TATAAT (Darnell, *supra*) (Seq. ID No. _____). In a preferred embodiment, the promoter is the *tac* promoter, although other promoters, such as the *trc* promoter (Borel et al., *FEBS 324*:162, 1993) that also comprise operable fusions of the -35 region of the *trp* promoter and the -10 region of the *lac* promoter, are also preferred embodiments.

Other negatively regulated promoters are also suitable for use in the present invention, provided that such a promoter is are repressed when present in lower numbers in a cell than the given promoter's repressor molecule, and that an increase in the copy number of operators (*i.e.*, repressor binding sites) effectively titrates out the effects of the repressor molecules, thereby inducing transcription of the desired gene. One example of such a promoter is an unaltered *trp* promoter (Yansura and Henner, *Meth. Enz. 185*:54-61, 1990). A person having ordinary skill in the art in light of the present specification would be able to utilize other promoters in the vector constructs, methods and other aspects of the invention. Such a person, in light of the present specification,

would also be able of the make nucleotide substitutions or other changes within either the -35 region or the -10 region of a suitable promoter, such as those discussed above, to provide slightly different but fully functional regions that would come within the scope of the present invention. Whether a promoter is operable, *i.e.*, repressed in the absence of inducer and expressed in the presence of inducer, can be readily determined by a person of ordinary skill in the art in light of the present specification, by screening for the presence or absence of PHA and/or PHB (for example, by examining cells under a light microscope for the presence of PHA and/or PHB), or for the presence or absence of mRNA produced from the genes of the *phb* operon (for example by hybridization assay).

In a promoter useful in the present invention, initiation of transcription may be repressed by binding a repressor, such as the *lacI* gene product, to the operator, which is located between the promoter and the *phb* operon. In the presence of a chemical inducer, preferably isopropyl-β-D-thiogalactoside (IPTG), the repressor is converted to an inactive form, thereby permitting initiation of transcription from the promoter. Other suitable inducers will be apparent to a person having ordinary skill in the art, in light of the present specification. Such inducers may include glucose-β-galactoside (lactose), glucose-α-galactoside (melibiose), and other lactose analogues such as methyl-β-galactoside and methyl-β-thiogalactoside. (Jacob and Monod, *J. Mol. Biol.* *3*:318-356, 1961.)

In another preferred embodiment, the *phb* operon is the *Alcaligenes eutrophus phb* operon. Other PHA and/or PHB producing *phb* operons, such as those found in the prokaryotic organisms including *Azotobacter*, *Beigerinckia*, *Alcaligenes*, *Pseudomonas*, *Rhizobium*, *Rhodospirillum* and *Azotobacter beijerinckii*, are also acceptable and therefore are included within the scope of the present invention.

In a further aspect, the present invention provides the *phb* operon incorporated into a runaway replicon vector construct. A runaway replicon vector construct is a vector construct that can be controllably induced and that, upon induction, significantly increases its copy number in the cell. In a preferred runaway replicon vector construct, the copy number of the vector construct is controlled by temperature (Nordstrom and Uhlin, *Biotechnology 10*:661, 1992). In this preferred embodiment, the *repA* gene, which encodes a protein that is required for the initiation of plasmid replication, is under the control of the λ pR promoter. The λ cI857 gene encodes a heat-sensitive repressor that actively inhibits transcription from the λ pR promoter at low temperature, but that is

inactive at high temperatures. Therefore, the incorporation of the λ cI857 gene in a host cell permits repression of the λ pR promoter at a low temperature. Thus, at low temperatures, such as 30°C, the vector construct copy number is low, while at high temperatures, such as 42°C, synthesis of *repA* mRNA increases, and the vector construct copy number is high.

In a further preferred embodiment, the runaway replicon vector construct further comprises a *tac* promoter operably linked to a *phb* operon, to provide a *tac::phb* fusion runaway replicon vector construct. The *tac::phb* fusion runaway replicon construct is a novel expression system in which the copy number and transcription of the *phb* operon are *both* efficiently controlled by temperature, even when IPTG (or other derepressor or inducer) is not present. At low temperatures, the copy number of the vector construct is lower than the number of lacI repressor proteins present in the cell (such number is typically about 5-10 proteins per cell (Muller-Hill et al., *Proc. Natl. Acad. Sci. 59*:1259, 1968). Thus, the number of lacI repressor molecules is sufficient to substantially repress transcription of the *phb* operon. At higher temperatures (which induce expression of the *repA* gene and therefore increase the copy number of the vector construct), the number of vector constructs surpasses the number of lacI repressor molecules. This rapidly results in non-repressed *phb* operons, and provides the highly advantageous, and unexpected result, that the *phb* operons are then expressed without the addition of an inducer such as IPTG. This system also provides the highly advantageous, and unexpected, result that the system is stable without the selective pressure of antibiotics to retain the vector construct (although the system preferably includes a stabilization locus, discussed further below, this advantageous and unexpected result may also be found in systems without a stabilization locus).

In yet another preferred embodiment, the present invention provides vector constructs as described above further comprising a stabilization locus. Suitable stabilization loci include *parB* (Gerdes, K., *Bio/Technology 6*:1402-1405, 1988), *ccd*, which appears to operate by a mechanism that involves post-segregational mortality of cells that lose a plasmid carrying the *ccd* locus (Gerdes, *supra*), the *pemK/pemI* system (Tsuchimoto, S. et al., *J. Bact. 170*:1461-1466, 1988), which also appears to involve mortality of plasmid-free segregants, and the plasmid maintenance system found in the F factor and encoded by the *sopA*, *sopB*, and *sopC* genes (Ogura and Hiraga, *Cell 32*:351-360, 1983). The provision of such a stabilization locus promotes stability of a desired plasmid in a cell. Determination of other suitable stabilization loci, in light of

the present specification, that would be suitable for use with the vector constructs, methods and other aspects of the present invention is within the skill of the art.

In another aspect, the present invention provides vector constructs in which the *phbC* Shine-Dalgarno sequence (*i.e.*, the native *phbC* Shine-Dalgarno sequence) is supplemented or replaced with a consensus Shine-Dalgarno sequence, preferably the *lac* Shine-Dalgarno sequence. A Shine-Dalgarno sequence is a sequence located about 10 bases to the 5' side of the start codon (typically AUG) of an mRNA sequence. (Zubay, *Biochemistry*, 944-45, 1983.) In a preferred embodiment, the consensus Shine-Dalgarno sequence comprises AGGA, although other suitable Shine-Dalgarno sequences could be easily utilized by a person having ordinary skill in the art in light of the present specification. Determination of the effectiveness of a Shine-Dalgarno sequence is also well within the skill of the art in light of the present specification, for example by screening for mRNA copy number.

In the course of making the present invention, it was discovered that the *phbC* gene was under post-transcriptional control, which control prevented optimal production of the *phbC* gene product (PHB synthase). This prevented maximal use of both the high-production vector constructs described above and traditional vector constructs containing the *phb* operon. The provision of a consensus Shine-Dalgarno sequence removes or lessens such post-transcriptional control, thereby permitting an increase in the translation of the *phbC* gene, production of the *phbC* gene product, and therefore production of PHA. Prior to the instant invention, it was not known that use of a consensus Shine-Dalgarno sequence could provide such an advantage.

In preferred embodiments, the vector construct including the consensus Shine-Dalgarno sequence is incorporated within one or more of the vector constructs described above. However, the consensus Shine-Dalgarno sequence can also be advantageously used with traditional vector constructs and the native *phb* promoter.

In still a further aspect, the present invention provides methods of producing PHB utilizing the vector constructs described above. In one embodiment, such methods include elevating the temperature of a culture at a certain time point in order to maximize PHB production. When a culture is slow growing (such as on minimal media), the temperature is preferably elevated at a later time in the log phase of the growth curve. For a fast growing culture, the temperature is preferably elevated earlier in the log phase of the growth curve.

Determination of whether a culture is slow growing or fast growing will depend upon such factors as growth media, strain background, temperature, and aeration. In light of the present specification, determination of whether a culture is slow growing or fast growing and the preferred time at which to induce the culture involves routine experimentation well within the ordinary skill in the art. In another embodiment, such methods include the provision of an inducer or derepressor, such as IPTG, that induces high production of PHB from the vector constructs. Determination of appropriate times to increase the temperature and/or add an inducer or derepressor is well within the skill of the art in light of the present specification.

In still another aspect, the present invention provides prokaryotic host cells transformed by the vector constructs described above. Various prokaryotic host cells may be utilized within the context of the present invention. Generally, preferred prokaryotic host cells should have a well-characterized genetic system, including known cloning vectors and methods of genetic manipulation. They should also preferably grow well in minimal medium, ideally to a high cell density, without any special requirements (physical or physiological). Representative examples of such host cells include members of the Bacillaceae, Nocardiaceae, Streptomycetaceae, Pseudomonadaceae, Corynebacteria, and Enterobacteriaceae. In a preferred embodiment, the host cell is able to metabolize sucrose. Preferred host cells in the Family Enterobacteriaceae include *Escherichia*, *Citrobacter*, *Klebsiella*, *Enterobacter*, and *Serratia*, as well as *Zymomonas* and *Flavobacterium*, which are within the Enterobacteriaceae but of uncertain affiliation. Particularly preferred host cells include *E. coli*, *Klebsiella oxytoca*, and *Klebsiella aerogenes*. Preferred host cells in the Family Pseudomonaceae include *P. aeruginosa*.

With respect to *Klebsiella*, and particularly *K. aerogenes*, the present invention provides an advantageous and unexpected result that the overproduction may be induced by a temperature increase to generally about 32°C to about 35°C, typically about 32.5°C to about 34°C, and preferably to about 33°C. This result is unexpected because it was previously believed (prior to the instant invention) that the induction of *repA* required a temperature increase to at least 36°C, and preferably to 42°C or more. Further unexpectedly, it has been discovered a temperature increase typically above 33°C, and generally above 34°C, results in smaller cell size and decreased yields.

The above-described prokaryotes may be readily obtained from a variety of commercial sources including, for example, the American Type

Culture Collection (ATCC) (Rockville, Maryland). Alternatively, many of the above-described bacteria may be isolated from sources that are known by those of skill in the art to contain such prokaryotes, based upon techniques that are known in the art. (*See* Bergy's *Shorter Manual of Determinative Bacteriology*, Williams & Wilkins (pub.), John G. Holt (ed.), 8th edition, 1977.)

Once the host cell has been cultured under conditions and for a time sufficient to generate PHA, the PHA is preferably isolated from the host cell. Isolation may be accomplished by a variety of methods. For example, the host cells may be lysed, and PHA agglomerated, essentially as described in U.S. Application Serial No. 07/528,549. Alternatively, lysozyme plasmids may be introduced into the host cell, and thereby utilized to enhance isolation of PHA, essentially as described in U.S. Application Serial No. 07/890,925.

Within a preferred embodiment, after the host cells have reached the stationary phase of growth, they are washed once with water to remove debris. The cells are then heat sterilized, and while still hot, SDS (approximately 0.1%) and EDTA (approximately 2mM) are added, and the mixture is stirred for about one hour at a temperature of 60°C to 80°C. During this time, the cells will lyse, releasing the PHA granules. The granules are separated from cell debris by centrifugation, and then washed twice with water.

Through use of the above-described techniques, PHA (or PHB) may be isolated to approximately 98% or 99% purity, as determined by gas chromatography. Briefly, PHA purity may be calculated by determining the area under the PHA peak, and dividing it by the areas under all peaks in the chromatogram.

EXAMPLES

Summary of the Examples

Generally, Examples 1-10, 12 and 13 are directed toward the construction of desired nucleic acid vectors. Examples 11 and 14-19 are directed toward assays for the effectiveness of various aspects of the present invention.

Example 1 is directed toward the construction of plasmid pJM9002 by inserting the *phb* operon-containing gene fragment from plasmid pJM8801 (previously designated p4A) into pBluescript SK^+.

Example 2 is directed toward the construction of plasmid pTZ18U-4c by deleting a segment containing the *phb* genes from plasmid pJM8703, which is also known as pTZ-18U-PHB.

Example 3 is directed toward the construction of plasmid pJM8905 by transferring a *phb* operon-containing fragment from pJM8703 into pSP72 to create pSP72/PHB, followed by excision of the *phb* fragment from pSP72/PHB and inserting it into pGEM-$7Zf^+$.

Example 4 is directed toward the construction of plasmid pJM9131 by the insertion of kanamycin resistance into, and the deletion of ampicillin resistance from, pJM8801.

Example 5 is directed toward the construction of plasmid pJM9117 by the insertion of the *phb* operon-containing fragment from pJM8703 into pRA89.

Example 6 is directed toward the construction of plasmid pJM9226 by the deletion of the *phb* operon-containing fragment from pJM9002 and the insertion of the *tac* promoter into pJM9002.

Example 7 is directed to the creation of *tac::phb* fusion plasmids pJM9227-pJM9229 by inserting the *phb* operon-containing fragment from pJM8905 into the *tac* promoter-containing pJM9226. pJM9227 has only ampicillin resistance, pJM9228 has both ampicillin resistance and kanamycin resistance, and pJM9229 has only kanamycin resistance.

Example 8 is directed toward the construction of *tac::phb* fusion plasmids pJM9230-pJM9232 by the insertion of the *phb* operon-containing fragment from pJM8703 into pJM9226. pJM9230 has only ampicillin resistance, pJM9231 has both ampicillin and kanamycin resistance, and pJM9232 has only kanamycin resistance. pJM9230-pJM9232 differ from pJM9227-pJM9229 in that pJM9230-pJM9232 have a *phbC* leader of approximately 355-bases that

contains a *cis*-acting element, while pJM9227-pJM9229 have a *phbC* leader of approximately 72 base pairs without such an element.

Example 9 is directed to the construction of runaway replicon *tac::phb* fusion plasmids pJM9233-pJM9236 by the insertion into the runaway replicon vectors pRA89 and pRA90 the *tac::phb* fusion from pJM9227. Thus, pJM9233-pJM9236 have both a tac promoter and a heat inducible promoter (λ pR). These plasmids differ from each other in the orientation and precise placement of the *phb* gene fragment within the vector.

Example 10 is directed to the construction of runaway replicon *tac::phb* fusion plasmids pJM9237 and pJM9238, which have an approximately 355-base leader.

Example 11 is directed to a graphic analysis of *phb* operon gene products, which analysis indicates that the *phbC* gene product (PHB synthase) is subject to post-translational regulation, and therefore is not overproduced by the plasmids constructed pursuant to Examples 1-10.

Examples 12 and 13 are directed to the construction of plasmids pJM9375 and pJM9376, which were created by the addition of a consensus Shine-Dalgarno sequence operably linked to the *phbC* gene. In pJM9375, the consensus (*lac*) Shine-Dalgarno sequence replaced the native *phbC* Shine-Dalgarno sequence. In pJM9376, the consensus (*lac*) Shine-Dalgarno sequence was added to the native *phbC* Shine-Dalgarno sequence.

Example 14 is directed to a graphic comparison of PHB production in native versus *tac* promoter clones.

Example 15 is directed to a graphic comparison of PHB production in the approximately 72 base pair leader and 355 base pair leader *phbC tac::phb* fusion constructs.

Example 16 is directed to the optimization of PHB production at different temperatures using the heat-inducible plasmid pJM9238.

Example 17 is directed to the determination of the optimal cell density during the cell growth cycle for initiation of PHB production using the plasmid pJM9238.

Example 18 is directed to a comparison of PHB production using the plasmid pJM9238 with or without chloramphenicol.

Example 19 is directed to the quantitation of PHB production in plasmids pJM9375 and pJM9376, each of which contain a *tac* promoter and a consensus (*lac*) Shine-Dalgarno sequence.

Example 20 is directed to the determination of the stability of PHB-producing plasmids in *Klebsiella*.

Example 21 is directed to the production of PHB in *Klebsiella* at varying temperatures.

Example 22 is directed to the production of PHB in *Klebsiella* using plasmid pJM9238 based on fed-batch fermentation. Example 22 is also directed to a comparison of PHB production using plasmid pJM9238 versus plasmid pJM9131.

Example 1. Construction of plasmid pJM9002.

The *phb* operon fragment was cloned into pBluescript SK^+ (Stratagene) as follows. In separate tubes, plasmid pJM8801 (previously designated p4A in U.S. Application Serial No. 07/890,925) and the vector pBluescript SK^+ were digested with the restriction endonucleases *Eco*R I and *Hind* III (Gibco BRL) as described (Maniatis et al., *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.). The released fragment containing the *phb* genes from plasmid pJM8801 was ligated into the *Eco*R I-*Hind* III digested pBluescript SK^+ fragment using T4 DNA Ligase (Gibco BRL) as described in Gibco Focus Technical Bulletin 5224-1. The resulting recombinant plasmid was designated pJM9002 (Figure 2).

Example 2. Construction of plasmid pTZ18U-4c

The plasmid pTZ18U-4c was constructed as follows. Plasmid pTZ-18U-PHB (deposited with American Type Culture Collection and assigned ATCC Deposit No. 299006, currently designated as pJM8703 (Figure 3), was digested with *Sph* I (which yields a 3' overhang) and *Bam* HI (which creates a 5' overhang). The resulting linearized fragment containing the *phb* genes was deleted from the *Bam* HI end using the procedure of Henikoff (Henikoff, S., Gene 28, 351, 1984) to approximately base 835 in the *phb* operon sequence previously disclosed (U.S. Application Serial No. 07/705,806). The fragment was then religated using T4 DNA Ligase and the resulting circularized plasmid was designated pTZ18U-4c (Figure 3).

Example 3. Construction of plasmid pJM8905

Plasmid pJM8905 was constructed as follows. The vector pJM8703, discussed above with respect to Figure 3, was linearized by digestion with *Eco* RI. The linearized plasmid DNA was then partially digested with *Pst* I as follows. From a 100µl digestion reaction, performed as described (Maniatis, *supra*), 10 µl aliquots were removed every 2.0 minutes to microplate wells containing 2 µl of 150 mM EDTA on ice. A total of 12 time-points were taken. Three microliters of 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose) were added to each well. Samples were separated on a 1% SeaKem GTG Agarose gel at 75 v for 30 minutes and visualized by ethidium bromide staining. The 11, 13, and 15 minute timepoints were selected because they contained the approximately 3.5 kb *Pst* I-*Eco*R I *phb* fragment, which was excised from the gel. To each gel slice was added plasmid pSP72 (Promega) that had been digested with *Pst* I and *Eco*R I. The mixture was then purified using GENECLEAN® (BIO101) according to the manufacturer's protocol. The eluted DNA was ligated using T4 DNA Ligase (Gibco BRL) as described above, and transformed into *E. coli* strain DH5α (*endA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1 Δ(lacZYA-argF)U169* (ϕ *80dlacd(lacZ)M15)*). The resulting plasmid was pSP72 containing the *Pst* I-*EcoR* I *phb* fragment in the multiple cloning site. This plasmid, designated pSP72/PHB (Figure 3), was next digested with *Eco*R I and *Xho* I. This released the *phb* fragment, which was then ligated into the same restriction sites of *Xho* I - *Eco*R I digested pGEM-7Zf$^+$ (Promega). The resulting plasmid was designated pJM8905 (Figure 3).

Example 4. Construction of plasmid pJM9131

The Kanamycin Resistance GENBLOCK® (*Eco* RI) (Pharmacia) restriction fragment was ligated into the *Eco* RI site of plasmid pJM8801 (p4A) (Janes et al., *supra*). The plasmid was then digested with *Dra* I and ligated to delete a restriction fragment within the *bla* gene. The resulting plasmid was designated pJM9131 (Figure 2).

Example 5. Construction of runaway replicon vector construct pJM9117

The *phb* fragment was cloned into pRA89 (Benzon Pharma A/S, Helseholmen 1, P.O. Box 1185, DK-2650 Hvidovre, Denmark; Figure 3) as follows. Plasmid p8703 (pTZ-18U-PHB) was digested with *Eco* RI. The resulting DNA fragment was partially digested with *Pst* I and the approximately 5 kb fragment containing the entire *phb* fragment was ligated using T4 DNA Ligase (Gibco BRL) into plasmid pSP73 (Promega) that had been digested with *Eco* RI and *Pst* I. The recombinant plasmid was transformed into *E. coli* strain DH5α. This plasmid was purified by the alkaline-lysis method (Maniatis et al., *supra*). The plasmid was then digested with *Xho* I and *Eco*R I to release the DNA fragment containing the *phb* operon. This fragment was ligated using T4 DNA Ligase into plasmid pGEM-7Zf$^+$ (Promega) that had been digested with *Xho* I and *Eco*R I. The resulting plasmid was designated pGEM7f-PHB reverse. Plasmid pGEM7f-PHB reverse was digested with *Bam*H I. The released *phb* fragment was ligated using T4 DNA Ligase into the *Bam* HI site of *Bam* HI-digested pRA89, which had been treated with Calf Intestinal Alkaline Phosphatase (Boehringer Mannheim). The resulting plasmid, designated pJM9117 (Figure 3), was introduced into *E. coli* strain HMS174 *recA1 hsdR Rif*^{r} by electroporation as previously described (see U.S. Application Serial No. 08/035,433).

Example 6. Construction of the *tac* promoter plasmid pJM9226

The *tac* promoter was cloned into the plasmid pBluescript SK$^+$ (Stratagene) as follows. The vector pJM9002 (described above) was digested with the restriction endonucleases *Bam*H I and *Hind* III (Gibco BRL) as described (Maniatis et al., *supra*). This released the *phb* fragment from the pBluescript plasmid. The *tac* promoter GENBLOCK® (*Hind* III/*Bam*H I) (Pharmacia) restriction fragment was ligated into the *Hind* III-*Bam*H I-digested pBluescript fragment using T4 DNA Ligase (Gibco BRL) as described in Gibco BRL Focus Technical Bulletin 5224-1.

The recombinant plasmid was introduced into *E. coli* strain DH5α by electroporation (Miller, *Bacterial Electroporation, Molecular Biology Reports No. 5*, Bio-Rad Laboratories, Richmond, CA, 1988) using the GENE PULSER® (Bio-Rad). Electroporation was performed as follows. An isolated

colony of *E. coli* strain DH5α was inoculated into a 13 x 100 mm S/P diSPo culture tube (Baxter) containing 3 ml of Luria-Bertani (LB) medium (Maniatis et al., *supra*). This culture was grown overnight in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) at 37°C, 225 rpm. The next morning 1 ml of the culture was inoculated in 50 ml of LB medium in a baffled 250 ml Erlenmeyer flask (Wheaton). Growth of the culture was followed by withdrawing aliquots at regular time intervals and measuring the optical density at 600 nanometers using a Shimadzu UV 160 U Spectrophotometer. The culture was incubated at 37°C, 225 rpm, until the optical density at 600 nanometers reached approximately 0.5. At this time, the culture was placed on ice for 10 minutes. It was then transferred to a sterile 50 ml Falcon 2098 Blue Max conical centrifuge tube and centrifuged in a Varifuge RF (Heraeus Instruments) at 3,000 g for 10 minutes. The supernatant was aseptically removed and 40 ml of sterile ice-cold 10% glycerol in deionized water was added to the pellet. The pellet was resuspended by vortexing, followed by pelleting as above. The supernatant was aseptically aspirated, 40 ml of sterile ice-cold 10% glycerol was again added and the pellet was resuspended. The bacteria were again pelleted by centrifugation as before and the supernatant was aseptically aspirated. Ten ml of sterile ice-cold 10% glycerol was added, the pellet was resuspended, and centrifuged as described above. The supernatant was aseptically aspirated and the pellet was resuspended in a final volume of approximately 200 µl. Forty microliter aliquots of this suspension were used for electroporation. One microliter of plasmid DNA was added to 40 µl of the cell suspension and this mixture was added to a 0.2 cm electrode gap GENE PULSER®/*E. coli* Pulser Cuvette (Bio-Rad Laboratories). The mixture was subjected to a pulse of 2.5KV, at 200 Ohms and 25 µfarads using a GENE PULSER® (Bio-Rad Laboratories). The bacterial suspension was then transferred to a sterile 13 x 100 mm culture tube containing 3 ml of LB broth. The culture was incubated for 1 hour in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) at 37°C, 225 rpm. Transformants were selected by spreading the cells on LB agar plates containing 200 µg/ml ampicillin (Sigma). The plates were incubated at 37°C in a Fisher IsoTemp Oven Model 350D until colonies were visible. Transformants were purified by picking well-isolated colonies and streaking for single colony isolation on LB agar plates containing 200 µg/ml ampicillin. Purified clones were inoculated into 3 ml of LB media containing 200 µg/ml ampicillin in 13 x100 mm culture tubes (Baxter) and incubated overnight at 37°C in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) at 200 rpm.

The plasmid DNA was purified by a modification of the alkaline lysis method as follows: A single colony was inoculated into a 13 x 100 mm culture tube containing 3 ml of LB broth + 200 µg/ml ampicillin and grown overnight. The culture was pipetted into two sterile 1.5 ml microfuge tubes (West Coast Scientific, Inc.) and pelleted in an Eppendorf Centrifuge 5415C microcentrifuge for two minutes. The supernatant was decanted. Each pellet was resuspended in 1 ml of ice-cold SET butter (20% sucrose, 50 mM EDTA, 50 mM Tris, pH 8.0) and centrifuged two minutes as before. The supernatant was withdrawn with a pipette. The cells were resuspended in a total of 150 µl of SET buffer and placed on ice. Five microliters of RNase (Boehringer Mannheim) (10 µg/ml, boiled for 2 minutes) was added and the tube was vortexed. Three hundred-fifty microliters of 0.2 N NaOH/1.0% SDS was added and the tube was inverted several times. The tube was incubated on ice for 20 minutes. Two hundred-fifty microliters of ice-cold 3 M sodium acetate, pH 5.2, was added and the tube was inverted gently several times and incubated on ice for 20 minutes. The tube was centrifuged for 5 minutes and the supernatant was decanted into a sterile 1.5 ml microfuge tube. Three hundred-fifty microliters of phenol (equilibrated with 0.1 M Tris buffer) (United States Biochemical Corp.) was added, the tube was vortexed, then 350 µl of chloroform was added and the tube was vortexed. The tube was centrifuged for 5 minutes in a Eppendorf Centrifuge 5415C microcentrifuge at room temperature. The top aqueous phase was transferred to a sterile microfuge tube (West Coast Scientific, Inc.). An equal volume (approximately 650 µl) of isopropanol was added, the tube was inverted and incubated at room temperature for 30 minutes. The tube was then centrifuged for 15 minutes in a microfuge at room temperature. The supernatant was removed, 500 µl of ice-cold 70% ethanol was added, and the tube was centrifuged in a Eppendorf Centrifuge 5415C microcentrifuge for 5 minutes at room temperature. The supernatant was removed and the pellet was dried. The dry pellet was resuspended in 10 µl of sterile water.

To confirm the presence of the *tac* promoter fragment in the vector construct, the DNA was digested with *Bam*H I (Gibco BRL) and *Hind* III (Gibco BRL). The restriction fragments were separated by gel electrophoresis on a 1.0% SeaPlaque (FMC BioProducts) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) using a BRL Horizon Model 58 Horizontal Gel Electrophoresis System and a BRL Model 200 Power Supply. The DNA was stained by placing the gel in a 0.5 µg/ml solution of ethidium bromide for 20 minutes, followed by destaining the gel in water for 30 minutes. The DNA was

visualized using a UV transilluminator (Fotodyne). This plasmid, designated pJM9226 (Figure 2), was subsequently used to construct *tac* promoter fusions to the *phb* operon. The construction of these fusions is described below.

Example 7. Construction of the multicopy *tac::phb* fusion plasmids pJM9227 and pJM9229 containing a *phbC* leader of approximately 72 base pairs

Plasmid pJM8905 (Figures 3 and 4) was digested with *Bst*B I (New England Biolabs), which cleaves 31 bp upstream of the *phbC* structural gene. T4 DNA Polymerase (Gibco BRL) was used to remove the single stranded DNA from the ends. Phosphorylated *Bam*H I linkers (New England Biolabs) were ligated to the blunt ends using T4 DNA Ligase (Gibco BRL). The resulting DNA was digested with *Bam*H. This released a *Bam*H I fragment that contained the *phb* structural genes but did not contain the promoter/regulatory region. The DNA restriction fragments were separated by preparative gel electrophoresis on a 1.0% SeaPlaque (FMC BioProducts) agarose gel in TAE buffer using a BRL Horizon Model 58 Horizontal Gel Electrophoresis System and a BRL Model 200 Power Supply. The DNA was visualized by staining with ethidum bromide. The restriction fragment containing the *phb* genes was excised from the gel and purified using GENECLEAN® (BIO101) according to the manufacturer's protocol. This fragment was then ligated into the *Bam*H I site of plasmid pJM9226 (Figures 2 and 4). The recombinant plasmid was then introduced into *E. coli* strain DH5α by electroporation and the transformants were spread on LB agar plates containing 100 μg/ml ampicillin and 1.0% glucose. The plates were incubated at 37°C in a Fisher IsoTemp Oven Model 350D until colonies were visible. White (PHB^+) colonies were picked and streaked for single isolates. Purified clones were inoculated into 3 ml of LB media containing 200 μg/ml ampicillin and grown to saturation. Plasmid DNA was prepared from the cultures as described above and digested with *Bam*H I and *Hind* III to confirm the *tac::phb* fusion construct. The resulting plasmid was designated pJM9227 (Figure 4).

This pJM9227 vector construct contains the *tac* promoter fused 78 bp upstream of the *phbC* structural gene. The native *phbC* Shine-Dalgarno sequence and ribosome-binding site is retained in this fusion. Thus, expression

of the *phb* genes is transcriptionally regulated by the *tac* promoter and translationally regulated by the native *phbC* Shine-Dalgarno sequence.

This construct proved to be unstable in liquid media. We believe this is because cells containing the plasmid excreted ampicillinase, which inactivated the ampicillin. Once this occurred the selective pressure to retain the plasmid was eliminated. Since high levels of expression of the *phb* genes were deleterious to the cell, plasmidless cells were rapidly selected. For these reasons, a different antibiotic resistance determinant was cloned into a restriction site downstream of the *phb* operon to increase plasmid stability. The plasmid pJM9227 was digested with *Eco*R I (Gibco BRL) and the 5' phosphate groups were removed using Calf Intestinal Phosphatase (New England Biolabs) to prevent self-ligation. The Kanamycin Resistance GENBLOCK® (*Eco* RI) (Pharmacia) restriction fragment was ligated into the *Eco*R I site located downstream of the *phb* genes. The resulting plasmid, pJM9228 (Figure 4), was introduced into *E. coli* strain DH5α by electroporation as previously described. The transformants were spread on LB agar plates containing 50 μg/ml kanamycin (Sigma). The plates were incubated at 37°C in a Fisher IsoTemp Oven Model 350D until colonies were visible. Plasmid DNA was isolated from purified clones. The plasmid-encoded ampicillin resistance gene was inactivated by *Dra* I (Gibco BRL) digestion to delete a 0.71 kb fragment from the *bla* structural gene, followed by religation. The resulting plasmid was designated pJM9229 (Figure 4).

This plasmid construct contains the *tac* promoter fused 78 bp upstream of the *phbC* structural gene, and results in a leader of approximately 72 bases (see Figure 1, panel b; Seq. ID No. _____). The native *phbC* Shine-Dalgarno sequence is retained in this fusion. Thus, expression of the *phb* genes is transcriptionally regulated by the *tac* promoter and translationally regulated by the native *phb* ribosome binding sites.

Example 8. Construction of multicopy *tac::phb* fusion plasmids pJM9230, pJM9231 and pJM9231 containing a *phbC* leader of approximately 355 base pairs

Plasmid pJM9226 (Figures 2 and 5) was digested with *Bam*H I and the 5' recessed ends were filled in with T4 DNA Polymerase to form blunt ends. A restriction fragment containing the *phb* operon was fused to the *tac* promoter

as follows: The plasmid pTZ18U-4c was digested with *Eco*R I and *Hind* III. This released a restriction fragment containing the *phb* structural genes and 290 bp of the upstream leader sequence. The ends of the fragment were filled in using T4 DNA Polymerase (Gibco BRL). The fragment was then excised from an agarose gel and purified using GENECLEAN®. The *phb* fragment was ligated into the plasmid containing the *tac* promoter fragment at the filled-in *Bam*H I site. The resulting plasmid, designated pJM9230 (Figure 5), was introduced into *E. coli* strain DH5α by electroporation as previously described. The transformed cells were plated on LB agar plates containing 200 μg/ml ampicillin. The presence of the *tac::phb* operon fusion was confirmed by screening for the PHB^+ phenotype (i.e., white colonies) on LB plates containing 200 μg/ml ampicillin and 1% glucose.

To increase plasmid stability, a kanamycin resistance gene was cloned into the plasmid as follows: The plasmid was digested with *Spe* I (New England Biolabs) and the recessed 5'-ends were filled-in with T4 DNA Polymerase to create blunt ends. The 5' recessed ends of a Kanamycin Resistance GENBLOCK® (*Eco* RI) (Pharmacia) restriction fragment were filled in with T4 DNA Polymerase. The Kanamycin Resistance GENBLOCK® was ligated into the filled-in *Spe* I site downstream of the *phb* operon. The recombinant plasmid, designated pJM9231 (Figure 5), was introduced into *E. coli* strain DH5α by electroporation and transformants were selected on LB agar plates containing 50 μg/ml kanamycin. Single clones were isolated and plasmid DNA isolated from these clones was purified as described above. The ampicillin resistance gene (*bla*) was inactivated by digestion with *Dra* I, followed by religation, which removed a 0.71 kb fragment from the structural gene. The resulting plasmid was designated pJM9232 (Figure 5). This construct contains the *tac* promoter fused 361 bp upstream of the *phbC* structural gene, resulting in a leader of approximately 355 bases (see Figure 1, panel c; Seq. ID No. _____). The native *phbC* Shine-Dalgarno sequence and ribosome binding site are retained.

Example 9. Construction of runaway replicon *tac::phb* fusion plasmids pJM9233, pJM9234, pJM9235 and pJM9236 containing a *phbC* leader of approximately 72 base pairs

The runaway replicon vector pRA89 (Benzon Pharma A/S, Helseholmen 1, P.O. Box 1185, DK-2650 Hvidovre, Denmark) (Figures 3 and 6) was digested with *Bam*H I and the ends were dephosphorylated using Calf Intestinal Phosphatase as described above. The *tac::phb* fusion was cloned into this vector as follows: The plasmid pJM9227 (Figures 4 and 6) was digested with *Eco*R I and *Hind* III and the single stranded ends were removed using T4 DNA Polymerase. The restriction fragments were separated by preparative gel electrophoresis on a 1.0% SeaPlaque (FMC BioProducts) agarose gel in TAE buffer using a BRL Horizon Model 58 Horizontal Gel Electrophoresis System and a BRL Model 200 Power Supply. The DNA was stained with 0.5 μg/ml ethidum bromide for 20 minutes and destained in water for 20 minutes. The bands were visualized using a UV transilluminator. The restriction fragment containing the *tac::phb* fusion was excised from the gel and purified using GENECLEAN® (BIO101) according to the manufacturer's protocol. Phosphorylated *Bam*H I linkers (New England Biolabs) were ligated to the ends of the fragment using T4 DNA Ligase and the DNA fragment was purified using GENECLEAN® (BIO101). This DNA fragment was then ligated into the *Bam*H I-digested vector pRA89. The recombinant plasmid was introduced into *E. coli* strain XL1-Blue F'::Tn*10* (p*roA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15/recA1 endA gyrA96* (Nal^{r}) *thi hsdR17* (*r*$_{k}^{-}$*m*$_{k}^{+}$) *supE44 relA1 lac*) by electroporation as previously described except the transformed cells were incubated at 30°C to allow expression of the plasmid-encoded antibiotic resistance factors. The transformants were spread on LB agar plates containing 25 μg/ml chloramphenicol (Sigma). The plates were incubated in a Fisher IsoTemp Oven Model 350D at 30°C to prevent runaway replication. Transformants were purified by picking well-isolated colonies and streaking for single colony isolation on LB agar plates containing 25 μg/ml chloramphenicol. The plates were incubated at 30°C to prevent runaway replication. The purified isolates were screened for the presence of the *phb* operon by streaking the isolates onto LB agar plates containing 25 μg/ml chloramphenicol + 1.0% glucose. The plates were incubated in a Fisher IsoTemp Oven Model 350D at 37°C to induce runaway replication. White colonies indicated the production of PHB, and thus the presence of the *phb* genes on the plasmid. Recombinants that exhibited a

PHB$^+$ phenotype at 37°C were inoculated into LB media containing 25 µg/ml chloramphenicol. The culture was incubated at 30°C for 6 hours, then 100 µl of the culture was used to inoculate 3 ml of LB media containing 50 µg/ml chloramphenicol. The cultures were incubated at 37°C overnight. Plasmid DNA was isolated from the cultures and digested with *Kpn* I (New England Biolabs) to determine the orientation of the *tac::phb* insert. The plasmid containing the *tac::phb* operon fusion with the *tac* promoter proximal to the chloramphenicol resistance gene (*cat*) was designated pJM9233 (Figure 6). The plasmid containing the *tac::phb* operon fusion with the *tac* promoter proximal to the *cI857* gene was designated pJM9234 (Figure 6). These *tac::phb* fusions were also cloned into the vector plasmid pRA90 (Benzon Pharma A/S, Helseholmen 1, P.O. Box 1185, DK-2650 Hvidovre, Denmark) (Figure 7) by the same procedure. The plasmid containing the *tac* promoter proximal to the *parB* locus was designated pJM9235 (Figure 7). The plasmid containing the *tac* promoter proximal to the chloramphenicol resistance gene (*cat*) was designated pJM9236 (Figure 7).

As described above for plasmid pJM9227, these plasmid constructs contain the *tac* promoter fused 78 bp upstream of the *phbC* structural gene, resulting in an approximately 72 bp leader (see Figure 1, panel b; Seq. ID No. _____). These plasmids retain the native *phbC* Shine-Dalgarno sequence and ribosome binding site.

Example 10. Construction of runaway replicon *tac::phb* fusion plasmids pJM9237 and pJM9238 containing a *phbC* leader of approximately 355 base pairs

The runaway replicon vector pRA90 (Benzon Pharma A/S, Helseholmen 1, P.O. Box 1185, DK-2650 Hvidovre, Denmark) (Figures 7 and 8) was digested with *Bam*H I and the 5' recessed ends were filled in with T4 DNA Polymerase. An *EcoR* I-*Hind* III restriction fragment from plasmid pJM9230 (Figures 5 and 8) containing the *tac* promoter and the *phb* structural genes was gel purified as previously described and the single-stranded ends were filled using T4 DNA Polymerase. This fragment was ligated into the filled-in *Bam*H I site of pRA90. The recombinant plasmid was introduced into *E. coli* strain XL1-Blue (Stratagene) by electroporation as previously described except the transformed cells were incubated at 30°C to allow expression of the plasmid-

encoded antibiotic resistance factors. The transformants were spread on LB agar plates containing 25 μg/ml chloramphenicol. The plates were incubated in a Fisher IsoTemp Oven Model 350D at 30°C to prevent runaway replication. Transformants were purified by single colony isolation and screened for the presence of the *phb* operon by streaking the isolates onto LB agar plates containing 25 μg/ml chloramphenicol and 1.0% glucose. The plates were incubated at 37°C to induce runaway replication. Recombinants that exhibited a PHB^+ phenotype at 37°C were inoculated into LB media containing 25 μg/ml chloramphenicol. The culture was incubated at 30°C for 6 hours, then 100 μl of the culture was used to inoculate 2.5 ml of LB media containing 50 μg/ml chloramphenicol. The cultures were incubated at 37°C overnight. Plasmid DNA was isolated from the cultures and digested with *Kpn* I to determine the orientation of the *tac::phb* insert. The plasmid containing the *tac::phb* operon fusion with the *tac* promoter proximal to the chloramphenicol resistance gene (*cat*) was designated pJM9237 (Figure 8). The plasmid containing the *tac::phb* operon fusion with the *tac* promoter proximal to the *cI857* gene was designated pJM9238 (Figure 8).

As described for plasmid pJM9230, these plasmid constructs contain the *tac* promoter fused 361 bp upstream of the *phbC* structural gene, resulting in a leader of approximately 355 bases (see Figure 1, panel c; Seq. ID No. _____). The native *phbC* Shine-Dalgarno sequence and ribosome binding site is retained.

Example 11. Analysis of the *phb* operon gene products by SDS-PAGE

The purpose of this experiment was to quantitate induction of the *phb* operon gene products in the *tac::phb* promoter constructs and to compare induction of the gene products to that observed in the native *phb* promoter clones. For this experiment, all of the plasmids were introduced into *E. coli* strain HMS174 by electroporation as previously described. To more effectively control expression of the *tac* promoter in this and other experiments, the plasmid pMS421 (obtained from G. Weinstock) (Figure 9), was introduced into the strains containing *tac::phb* fusions on multicopy plasmids by electroporation. This procedure was performed as previously described, except the transformants were selected on LB agar plates containing 10 μg/ml streptomycin. The plasmid pMS421 is a low copy number vector that confers streptomycin resistance and

contains the *lacI*^{q} gene, which overproduces the Lac repressor protein (Muller-Hill and Gilbert, *Proc. Natl. Acad. Sci. 59*:1259, 1968). Five *E. coli* strains were used in this experiment: HMS174, HMS174 pJM9131, HMS174 pJM9232 pMS421, HMS174 pJM9117 , and HMS174 pJM9238. *E. coli* strain HMS 174 was inoculated into 3 ml of LB medium. *E. coli* strain HMS174 pJM9131 was inoculated into 3 ml of LB medium containing 50 μg/ml kanamycin. *E. coli* strain HMS174 pJM9232 pMS421 was inoculated into 3 ml LB medium containing 50 μg/ml kanamycin and 10 μg/ml streptomycin. These cultures were shaken at 200 rpm at 37°C in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) for approximately 15 hours. *E. coli* strain HMS174 pJM9117 and *E. coli* strain HMS174 pJM9238 were each inoculated into 3 ml of LB medium containing 25 μg/ml chloramphenicol. These cultures were shaken at 200 rpm at 30°C in a Lab-Line Orbital Environ-Shaker (Lab-Line Instruments, Inc.) for approximately 15 hours. The cultures were diluted 1:100 into 50 ml of the same media in a 250 ml baffled Erlenmeyer flask (Wheaton), except glucose was added to a final concentration of 1.0%, and the cultures were incubated at the same temperature and agitation as previously described. The growth of the culture was followed by withdrawing aliquots at regular time intervals and measuring the optical density at 600 nanometers using a Shimadzu UV 160U Spectrophotometer. The *phb* operon was induced in *E. coli* strain HMS174 pJM9232 pMS421 by the addition of IPTG to a final concentration of 10 mM at an OD_{600} of 2.75. The *phb* operon was induced in *E. coli* strain HMS174 pJM9117 and *E. coli* strain HMS174 pJM9238 by transferring the cultures to a 41°C waterbath for 30 minutes when an OD_{600} of 0.7 was reached. A sterile stir bar was added and the cultures were mixed at 200 rpm using a Fisher Scientific Electronic Stirrer 2008. Following the heat pulse, the cultures were incubated in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) at 37°C and shaken at 200 rpm. One milliliter aliquots were withdrawn at time intervals, centrifuged in an Eppendorf Centrifuge 5415C and frozen at -70°C. These samples were subsequently analyzed by 1-D SDS-PAGE analysis. Proteins were separated by gel electrophoresis using precast Mini-PROTEAN II Ready Gels (Bio-Rad). A 12% polyacrylamide gel was used to resolve the thiolase and reductase proteins. The proteins were electrophoresed for approximately 45 minutes using the Mini-PROTEAN II® Electrophoresis Cell (Bio-Rad) and BRL Model 200 Power Supply. Prestained SDS-PAGE Standards (Bio-Rad) were used as molecular weight markers to monitor protein migration through the gels during electrophoresis. The proteins were visualized by silver staining using the Bio-

Rad Silver Stain Plus kit according to the manufacturer's protocol. The gels were dried between cellophane sheets using drying frames (Integrated Separation Systems). The *phb* gene products were quantitated as a relative percentage of total protein by densitometry using a Ultrascan XL Enhanced Laser Densitometer (LKB).

Results: The thiolase and reductase bands were clearly visible after induction in the samples from the *tac::phb* clones, while the synthase was undetectable in all samples. The *phb* gene products were not present in the negative control *E. coli* strain HMS174. In *E. coli* strain HMS174 pJM9131 (*phb* promoter, multicopy) the thiolase and reductase levels remain relatively constant. The thiolase levels were 1.61%-1.86% of total protein, and the reductase levels were 0.38%-0.95% of total protein (Figure 10, panel a). In *E. coli* strain HMS174 pJM9232 pMS421 (*tac* promoter, multicopy) the thiolase and reductase levels rapidly increased after the addition of IPTG. The thiolase level rose from 0.78% of total protein before induction to 4.36% 30 minutes after IPTG addition. The reductase levels increased from 0.73% to 1.63% of total protein 3 hours after IPTG addition (Figure 10, panel b). In *E. coli* strain HMS174 pJM9117 (*phb* promoter, runaway replicon) the thiolase and reductase proteins were undetectable before induction. The thiolase level increased to 2.54% and the reductase level rose to 10.11% of total protein 2.3 hours after the heat pulse (Figure 10, panel c). In *E. coli* strain HMS174 pJM9238 (*tac* promoter, runaway replicon) The thiolase and reductase levels increased from undetectable levels to 15.2% and 9.34% of total protein, respectively, 2 hours after the heat pulse (Figure 10, panel d).

These results indicate that synthase production is regulated post-transcriptionally, since replacement of the *phbC* promoter with the *tac* promoter did not measurably increase the synthesis of the *phbC* gene product. Control of thiolase synthesis is clearly regulated by the *tac* promoter. In both *tac::phb* fusion constructs, this protein was rapidly synthesized to relatively high levels following induction. In contrast, reductase synthesis appears to be related to plasmid type or copy number. In the multicopy plasmids, the highest reductase levels are 0.95% for *E. coli* strain HMS174 pJM9131 and 1.63% for *E. coli* strain HMS174 pJM9232 pMS421. In contrast, the runaway replicon plasmids have significantly higher levels of reductase. The highest reductase level for *E. coli* strain HMS174 pJM9117 was 11.55% of total protein. For *E. coli* strain HMS174 pJM9238, the highest reductase level was 10.01% of total protein.

In summary, synthase production appears to be primarily regulated at the post-transcriptional level. Synthesis of thiolase is regulated at the level of transcription. Synthesis of reductase appears to be a function of plasmid type or copy number, and may be regulated by a gene dosage effect.

Example 12. Replacement of the native *phbC* ribosome-binding site with the *lac* ribosome-binding site to create plasmids pJM9375 and pJM9376

1-D SDS-PAGE analysis of the *phb* gene products indicated that the *phbC* gene product was not induced to detectable levels in either the native promoter clones or the *tac::phb* fusions. This indicated that this gene is post-transcriptionally regulated. Inspection of the *phb* operon DNA sequence indicates that the *phbA* and *phbB* genes are each preceded by a putative Shine-Dalgarno sequence that perfectly or nearly matches, respectively, the consensus sequence 5'AGGAG 3' (Gold, *Ann Rev. Biochem. 57*:199-233, 1988); however, a Shine-Dalgarno sequence similar to that of the consensus could not be found in the *phbC* ribosome-binding region. Inefficient translation of the *phbC* gene would result in very low levels of the synthase even under fully induced conditions. In order to more efficiently translate the *phbC* gene, the native *phbC* ribosome-binding site was replaced with the *lac* ribosome-binding site. The *lac* Shine-Dalgarno sequences closely matches the consensus sequence and thus, ribosomes are predicted to have a higher affinity for this site than for the *phbC* Shine-Dalgarno sequence. It was hoped that this replacement would result in increased *phbC* synthesis, with a resulting increase in PHB yields.

The *phbC* Shine-Dalgarno sequence was replaced as follows. A 50 ml culture of *E. coli* strain XL-1 Blue (Stratagene) pJM9230 was grown to saturation in LB + 50 μg/ml kanamycin in a 250 ml Erlenmeyer flask (Wheaton). Plasmid pJM9230 was purified from the culture using the QIAGEN Plasmid Kit (QIAGEN Inc.) according to the manufacturer's protocol. This procedure is based on a modification of the alkaline lysis method (Birnboim and Doly, *Nucl. Acids. Res. 7*:1513-1522, 1979.). The plasmid was digested the *Bst*B I, which cleaves at a site approximately 30 bp upstream of the *phbC* structural gene. Small deletions were made from this site into the *phbC* ribosome-binding region with Exonuclease III using the double-stranded Nested Deletion Kit (Pharmacia) according to the manufacturer's protocol, with the following modifications: The

incubation temperature was 30°C and the digestion buffer contained 150 mM NaCl.

To measure the extent of the deletion, an aliquot of DNA from each time point was digested with *Not* I (New England Biolabs), which cleaves at a site within the *phbC* structural gene. The DNA was separated by gel electrophoresis in 3.0% Meta-Phor agarose (FMC) in TAE buffer using a BRL Model H5 Horizontal Gel Electrophoresis System and a BRL Model 100 Power Supply. The gel was stained in a solution of 0.5 μg/ml ethidium bromide for 20 minutes and destained in water for one hour. DNA samples in which the deletions extended into the *phbC* ribosome-binding site but not into the *phbC* structural gene were chosen for cloning.

Phosphorylated *Bam*H I linkers were ligated to the blunt ends of the deletion endpoints using T4 DNA Ligase and the DNA was introduced into *E. coli* strain XL-1 Blue cells by electroporation as previously described. Transformants were spread onto LB agar plates containing 200 μg/ml ampicillin and 1.0% glucose. Light-brown translucent (PHB^-) colonies were picked and purified by single colony isolation. These mutants were defective in PHB production, presumably because they were unable to synthesize the *phbC* gene product. Since the promoter region was still intact and the deletions did not extend into the *phbC* structural gene, this phenotype could only be due to a partial or complete deletion of the *phbC* ribosome-binding site. Several isolates were picked and purified by single colony isolation. These mutants were inoculated into 3 ml of LB liquid media containing 200 μg/ml ampicillin. The plasmid DNA was purified using the QIAGEN Plasmid Kit (QIAGEN Inc.) according to the manufacturer's protocol and digested with *Bam*H I. Linearization of the DNA indicated that the *Bam*H I linkers were successfully ligated to the endpoints of the deletion. An aliquot of the *Bam*H I-digested samples was digested with *Hind* III. This removed all DNA sequence upstream of the deletion endpoint including the promoter. The *tac* promoter GENBLOCK® restriction fragment was ligated into the *Hind* III-*Bam*H I pJM9230 fragment using T4 DNA ligase and the recombinant plasmid was introduced into *E. coli* strain HMS174 pMS421 by electroporation as previously described. The transformed cells were selected on LB agar plates containing 200 μg/ml ampicillin and 10 μg/ml streptomycin. Colonies were replica plated onto LB agar plates containing 200 μg/ml ampicillin + 1.0% glucose + 1 mM IPTG using an Accutran Replica Plater (Schleicher & Schuell). Deletion derivates that

yielded large white colonies were isolated from the original selection plates and purified. The plasmids were designated pJM9375 and pJM9376 (Figure 11).

Example 13: Determination of the *tac* GeneBlock-*phbC* leader fusion joint in plasmids pJM9375 and pJM9376 by sequence analysis.

The precise endpoints of the *Exo* III deletions were determined by sequence analysis. The plasmid DNA used as template was isolated from 50 ml cultures of *E. coli* strain HMS174 pJM9375 pMS421 and HMS174 pJM9376 pMS421 grown to saturation in LB media containing 200 µg/ml ampicillin + 10 µg/ml streptomycin. The plasmid DNA was purified using a QIAGEN Plasmid Kit (QIAGEN Inc.) as previously described. The DNA was sequenced using a Li-Cor DNA Sequencer Model 4000. The primer used was an infrared dye-labeled M13 17-mer -20 Sequencing Primer (3'TGACCGGCAGCAAAATG5') (Seq. I.D. No. _____). Sequenase Version 2.0 T7 DNA Polymerase (United States Biochemical) was used to extend the primer. The dideoxy reaction was performed as described in the Li-Cor Model 4000 Quick Start Tutorial (Li-Cor), Section 3, with the following modifications: no DTT was used, and the template and primer were annealed for 1 hour at 55°C.

The sequence of the clones is shown in Figure 1, panels d and e (Seq. ID Nos. _____ and _____). Previous studies (reviewed by Gold, *Ann Rev. Biochem. 57*:199-233, 1988) indicate that optimal spacing between the translational start codon and the Shine-Dalgarno sequence is greater than five nucleotides and less than thirteen. In the pJM9375 clone the spacing between the first (downstream) *lac* Shine-Dalgarno sequence and the start of the *phbC* structural gene, 4 bases, is less than optimal. The spacing between the *phbC* start codon and the second (upstream) *lac* Shine-Dalgarno sequence, 11 bases, is within the optimal range. In the process of constructing the pJM9375 clone a mutation was made that resulted in an alteration of the *phbC* start codon. The ATG start codon was replaced with a GTG start codon. Although some mRNAs exhibit the same translational yield with ATG and GTG, the ATG codon usually results in higher translation (Gold, *supra*). In the pJM9376 clone the spacing between the start of the *phbC* structural gene and the *lac* Shine-Dalgarno sequence, 18 bases, is greater than the optimal typical range. This construct retains the putative *phbC* Shine-Dalgarno sequence and the ATG start codon.

Example 14. Comparison of PHB production in native and *tac* promoter *phb* multicopy clones

The purpose of this experiment was to compare PHB production of the *tac::phb* fusion constructs with that of the native *phb* clones in the multicopy and runaway replicon systems. Four *E. coli* strains were used in this experiment: HMS174 pJM9131, HMS174 pJM9232 pMS421, HMS174 pJM9117, and HMS174 pJM9238. *E. coli* strain HMS174 pJM9131 was inoculated into 50 ml of LB medium containing 50 μg/ml kanamycin in a 250 ml Erlenmeyer flask. *E. coli* strain HMS174 pJM9232 pMS421 was inoculated into 50 ml LB medium containing 50 μg/ml kanamycin and 10 μg/ml streptomycin in a 250 ml Erlenmeyer flask. These cultures were shaken at 225 rpm at 37°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific) for approximately 15 hours. *E. coli* strain HMS174 pJM9232 and *E. coli* strain HMS174 pJM9238 were each inoculated into 50 ml of LB medium containing 25 μg/ml chloramphenicol in a 250 ml Erlenmeyer flask. These cultures were shaken at 225 rpm at 30°C in an Innova 4000 Incubator Shaker (New Brunswick Scientific) for approximately 15 hours. The cultures were diluted to a final optical density at 600 nm of 0.10 into 250 ml of the same media in a 1 liter baffled Erlenmeyer flask, except glucose was added to a final concentration of 2.0%, and the cultures were incubated at the same temperature and agitation as previously described. The growth of the culture was followed by withdrawing aliquots at regular time intervals and measuring the optical density at 600 nanometers using a Shimadzu UV 160U Spectrophotometer.

The *phb* operon was induced in *E. coli* strain HMS174 pJM9232 pMS421 by the addition of IPTG to a final concentration of 10 mM at an OD_{600} of 2.75. The *phb* operon was induced in *E. coli* strain HMS174 pJM9117 and *E. coli* strain HMS174 pJM9238 by transferring the cultures to a 41°C waterbath for 30 minutes when an OD_{600} of 0.7 was reached. (Previous experiments indicated that the optimal cell density for induction of *E. coli* strain HMS174 pJM9117 in minimal media is at an optical density of 1.0 or slightly lower. When cells were induced at cell densities slightly higher than 1.0, the PHB yield rapidly decreased.) A sterile stir bar was added and the cultures were mixed at 200 rpm using a Fisher Scientific Electronic Stirrer 2008. Following the heat pulse, the cultures were incubated in a 37°C in an Innova Incubator Shaker (New Brunswick Scientific) and shaken at 200 rpm.

Aliquots were withdrawn for dry weight determinations and to quantitate PHB production as follows. For the PHB assay, duplicate 3 ml samples were placed in Pyrex No. 9826 screw-capped tubes and pelleted by centrifugation in a Varifuge RF centrifuge (Heraeus Instruments) for 10 minutes at 2500 rpm. The supernatant was aspirated and discarded, and the tubes containing the cell pellets were placed at -70°C for at least one hour. Uncapped screw-capped tubes containing the frozen pellets were then placed in a Labconco lyophilizer for approximately 2 hours until samples were freeze-dried.

Samples were then subjected to methanolysis as follows. To each tube was added 1.7 ml ACS grade methanol (Mallinckrodt), 2 ml ACS grade chloroform (Mallinckrodt), 0.3 ml concentrated sulfuric acid (added while vortexing tube) and 0.1 ml benzoic acid solution (2 mg/ml). Samples were capped tightly, placed in a heat-block adjusted to 100°C and incubated for 140 minutes. Samples were then removed from the heat block and allowed to cool to room temperature. One ml of deionized water was then added to each tube, the tubes were vortexed for 30 seconds, and then centrifuged in a Varifuge RF centrifuge (Heraeus Instruments) for 10 minutes at 2500 rpm. The upper aqueous phase of each sample was removed by aspiration and the remaining organic phase was pipetted into vials and assayed for PHB production by gas chromatography.

The gas chromatography system consisted of a Shimadzu GC-14A, connected to a CR-4A data processing unit, an AOC-14 autoinjector, and an AOC-1400 autosampler. The carrier gas was UPC grade helium and detection was through a flame ionization detector. The flow rate of the carrier was approximately 5 ml/min. The column used for detection was a Supelcowax 10 column (Supelco Separation Technologies). The column is a 15 meter column, 0.53 mm inner diameter, with a 1 μm thick coating. Samples (1 to 3 μl) were injected into the injection port (temperature 200°C) and carried into the column. The samples were run under temperature profile of 55°C for 5 minutes, followed by a temperature ramp of 5°C per minutes until the column temperature reached 220°C. The temperature was held at 220°C for 5 minutes, followed by a termination of the run and cool-down for the next run. Typically, the solvent peak eluted through the detector (240°C) between 1 and 2 minutes, and the PHB peak eluted between 3 and 4 minutes. Analyses were done using benzoic acid (100 μl of 2 mg/ml solution in methanolysis tubes) as an internal standard. Typically, benzoic acid eluted from the GC column approximately 5 minutes into the run.

PHB samples were weighed out on Sartorius balance and subjected to methanolysis. The area under each curve (integration by Shimadzu data processor) was graphed against the known weights. The resulting line was used to generate an equation that could be used in calculating the PHB content in the experimental samples using the integration area under the PHB peak.

For dry weight determinations, 5.0 ml samples were removed from the cultures and centrifuged for 10 minutes at 3000 rpm in a Varifuge RF centrifuge (Heraeus Instruments). The supernatant was removed by aspiration and the cell pellet was resuspended in 1.0 ml of 0.85% saline solution. This was added to a preweighed aluminum weigh boat and placed in a 80°C drying oven for approximately 30 hours. The dry weight was calculated by subtracting the weight of the empty aluminum weigh boat and the weight of 1.0 ml of 0.85% saline from the total weight. This value was then divided by 5 to obtain the dry weight in mg/ml.

Glucose concentrations were quantitated using the Sigma Diagnostics Glucose Assay Kit (Sigma), Procedure No. 635, p. 5. Test tubes were labeled blank, standard, and test. To each blank tube, 0.1 ml water was added. To the standard tubes, 0.1 ml of diluted Glucose Standard solution (Catalog No. 635-100) were added at concentrations of 1-20 mM. One milliliter of each culture sample to be tested was centrifuged in a Eppendorf Centrifuge 5415C microcentrifuge for two minutes to pellet the cells. To each test tube, 0.1 ml of culture supernatant was added, then 5.0 ml of o-Toluidine Reagent (Catalog No. 635-6) was added. The tubes were mixed by vortexing and placed into a 100°C heat block for 10 minutes. The tubes were removed and cooled to room temperature. The contents of tubes were transferred to cuvettes and the absorbance at 635 nm was read using a Shimadzu UV 160U Spectrophotometer with the blank as reference.

The plasmid copy number was determined for each culture as follows. Two hundred microliters of cell suspension was centrifuged in a Eppendorf Centrifuge 5415C microcentrifuge for one minute. The supernatant was aspirated off and discarded. The cell pellet was resuspended in 50 ml of 10 mM Tris (pH 8.0), 10 mM EDTA, 100 mM NaCl, 20% sucrose, 1.5 mg/ml lysozyme (Sigma), 2 units/ml RNase. The solution was incubated for 30 minutes at 37°C. Fifty microliters of 2% SDS was added and the solution was mixed by vortexing at the maximum setting for two minutes. The solution was frozen at -70°C and thawed for two cycles. Five microliters of a 400 mg/ml proteinase K (BRL) stock solution was added and the tube was incubated for 30 minutes at

37°C. Twenty-five microliters of loading buffer (50% glycerol, 1 mM EDTA, pH 8.0, 0.1% bromophenol blue) was added and 5-15 µl of the sample was loaded on a 0.9% agarose gel in TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer. The gel was run for three hours at 75 volts. The gel was stained for 40 minutes in 1 µg/ ml ethidum bromide solution. The gel was then destained for 20 minutes in water, rinsed, and destained an additional 20 minutes. The gel was placed on a UV transilluminator (Fotodyne) and photographed using a Polaroid MP-4 Land Camera with Polaroid Type 665 film. The lowest F-stop was used and the shutter was opened for 45 seconds. Immediately after exposure, the negative was placed in fixer solution and agitated gently for approximately 30 seconds (in the dark). The negative was then washed under 65°C running water for 5 minutes and dried. The plasmid and chromosomal bands were quantitated by densitometry using an Ultrascan XL Enhanced Laser Densitometer (LKB) at the following settings: X-width=5, Beam=line, Offset=0, Y-step=1, Smoothing=1, X-step=250, A-axis=normalized, Baseline=2, Peak width=5,5,1. The copy number was calculated by the following formula:

$$\frac{\text{(area in plasmid band)/ (area in chromosome band)}}{\text{(kb in plasmid)/(kb in chromosome)}}$$

Results: The presence of the *tac* promoter in the runaway replicon vector significantly increased the induction rate and yield. In the *tac* promoter construct *E. coli* strain HMS174 pJM9238, the PHB concentration reached 3 mg/ml (72% of dry weight) approximately 8 hours after induction (Figure 12, panel a). At the same time after induction, the PHB concentration was less than 1.3 mg/ml (46% of dry weight) in the native promoter clone *E. coli* strain HMS174 pJM9117 (Figure 12, panel b) and 1.8 mg/ml (64% of dry weight) and 1.7 mg/ml (57% of dry weight) in *E. coli* strains HMS174 pJM9131 and HMS174 pJM9232 pMS421, respectively (Figure 12, panels c and d). *E. coli* strain HMS174 pJM9238 also retained a higher PHB yield as a percentage of dry weight than strain HMS174 pJM9117 throughout the post-induction period (Figure 13, panel a). This difference was not due to a higher gene dosage for the *tac* promoter clone. In fact, the pJM9117 copy number was slightly higher than the pJM9238 copy number (Figure 14, panel a). Neither clone utilized all of the glucose by 24 hours, indicating that the conditions of induction were not optimal for either strain in LB medium; however, the comparison is valid since the strains were induced under the same conditions.

In the multicopy plasmid constructs the induction kinetics and yields were not significantly different. In both strains PHB production continued to increase after induction. The yield at 23 hours was 5.2 mg/ml PHB (77% dry weight) for the native promoter clone *E. coli* strain HMS174 pJM9131 (Figure 12, panel c) and 5.6 mg/ml PHB (78% dry weight) for the *tac* promoter clone *E. coli* strain HMS174 pJM9232 pMS421 (Figure 12, panel d). Both strains had similar PHB yields as a percentage of dry weight after induction, although this value was significantly lower for strain HMS174 pJM9232 pMS421 before the addition of IPTG (Figure 13, panel b), indicating efficient regulation of *phb* expression by the *lac* operator-repressor system. Although the copy number of plasmid pJM9131 was higher than that of plasmid pJM9232 (Figure 14, panel b), this difference had no significant effect on PHB production or yield as a percentage of dry weight.

Example 15. Comparison of PHB production in the 78 bp leader and the 361 bp leader *phbC tac::phb* fusion constructs

As described in the section detailing plasmid constructions, two types of *tac::phb* fusions were constructed. In one type of fusion the *tac* promoter was inserted 78 bp upstream of the *phbC* structural gene. In the other type of fusion the *tac* promoter was inserted 361 bp upstream of the *phbC* structural gene. Each type of fusion was cloned into a multicopy vector and runaway replicon vectors. To determine if the leader sequence contained *cis*-acting elements that regulated the expression of the *phb* genes, PHB production was quantitated in *tac::phb* fusion multicopy clones containing each type of fusion. The *E. coli* strains used in this study were HMS174 pJM9229 pMS421 and HMS174 pJM9232 pMS421. The strains were inoculated into 50 ml of LB media containing 50 μg/ml kanamycin and 10 μg/ml streptomycin in a 250 ml baffled Erlenmeyer flask (Wheaton). The culture was incubated at 200 rpm at 37 °C in a Lab-Line Orbital Environ-Shaker (Lab-Line Instruments, Inc.) for approximately 15 hours. One ml of the stationary phase culture was added to 250 ml of LB media containing 1.0% glucose + 50 μg/ml kanamycin + 10 μg/ml streptomycin in a 1 liter baffled Erlenmeyer flask (Bellco) and incubated at 200 rpm at 37°C. The growth of the culture was followed by withdrawing aliquots at regular time intervals and measuring the optical density at 600 nanometers using a Shimadzu UV 160U Spectrophotometer. At an OD_{600} of 2.0, IPTG (United

State Biochemical Corp.) was added to each culture to a final concentration of 10 mM. Samples were withdrawn at regular time intervals for GC analysis.

Results: PHB was not produced in either strain until the addition of the chemical inducer IPTG. PHB production is rapidly induced after the addition of IPTG in both strains (Figure 15). The 361 bp leader *tac::phb* fusion construct produces more PHB than the 78 bp leader fusion construct. After 5 hours the strain containing plasmid pJM9232 produced twice as much PHB as the strain containing pJM9229, as indicated by GC counts. These results indicate that the leader sequence contains a *cis*-acting positive regulatory element or elements that increases *phb* expression. The results also show that the presence of the *lacIq* gene on plasmid pMS421 is able to very effectively repress transcription of the *phb* operon in both of these constructs.

Example 16: Optimization of PHB Production at Different Temperatures in *E. coli* strain HMS174 pJM9238

Preliminary experiments in which PHB production was compared in native and *tac* promoter *phb* clones (see Example 14, above) indicated that the conditions for induction were not optimal for *E. coli* strain HMS174 pJM9238. As one step to determine the optimal conditions for PHB synthesis, the strain was grown at a constant temperature and PHB production was quantitated. *E. coli* strain HMS174 pJM9238 was inoculated into 50 ml of LB + 25 µg/ml chloramphenicol and the culture was incubated overnight at 30°C. The next morning, 250 ml of LB media containing 2% glucose and 25 µg/ml chloramphenicol in a 1 liter baffled Erlenmeyer flask (Wheaton) was equilibrated to the proper temperature by placing the flask in an incubator. The optical density at 600 nm of the overnight culture was determined and enough volume of the culture was added to the medium to obtain an initial optical density at 600 nm of 0.10. The culture was incubated at a constant temperature of 30°C, 32°C, 34°C, 36°C, 38°C, or 40°C in an Innova 4000 Incubator Shaker (New Brunswick Scientific) at an rpm setting of 175. A total of 5 samples were taken for each culture in the optical density range of 0.4 to 2.0 and used to assay PHB and determine the dry weight as previously described.

Results: PHB levels were 0.1%-2% of the cell dry weight when the cultures were incubated at temperatures of 30°C, 32°C, and 34°C (data not shown). At 36°C the PHB concentration was 10% of the cell dry weight after

about 2 hours of incubation. The PHB concentration increased to over 40% of the cell dry weight after 1.5 hours of incubation at 38°C. At 40°C the PHB concentration was approximately 20% of cell dry weight after 2 hours of incubation (Figure 16). These results indicate that the culture should be incubated at 36°C during the growth phase and shifted to 38°C to initiate PHB production.

Example 17: Determination of the optimal cell density to initiate PHB production in *E. coli* strain HMS174 pJM9238

As a second step to increase PHB production, the optimal cell density at which to induce the PHB operon was determined to this, a culture of *E. coli* strain HMS174 pJM9238 was induced at various cell densities as follows: The strain was inoculated into 50 ml of LB + 25 µg/ml chloramphenicol and the culture was incubated overnight at 30°C. The next morning, 250 ml of LB media containing 2% glucose and 25 µg/ml chloramphenicol in a 1 liter baffled Erlenmeyer flask (Wheaton) was equilibrated to 36°C by placing the flask in an incubator. The optical density at 600 nm of the overnight culture was determined and enough volume of the culture was added to the medium to obtain an initial optical density at 600 nm of 0.10. The culture was incubated at a constant temperature of 36°C in a Innova 4000 Incubator Shaker (New Brunswick Scientific) at an RPM setting of 175. The growth of the culture was followed by measuring the optical density as previously described. Twenty milliliter aliquots of the culture were withdrawn at various times during growth and added to sterile 250 ml baffled Erlenmeyer flasks (Bellco) prewarmed to 38° C in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.). The cultures were incubated at 175 RPM for a total of 24 hours, after which the cells were harvested. PHB production and dry weight of each culture was determined as previously described.

Results: The results indicate that PHB production is highest when the culture is induced at low cell density. When the culture was induced at an optical density of 0.10, the 24 hour culture contained 5.5 mg/ml PHB (71% of dry weight). PHB production was lowest when the culture was induced at mid-log phase. When induced at an optical density of 0.78, the 24 hour culture contained only 2.2 mg/ml PHB (48% of dry weight). PHB production increased as the cells entered late log phase. When the culture was induced at an optical

density of 2.05, the 24 hour culture contained 3.7 mg/ml PHB (66% of dry weight) (Figure 17). These results are different from those obtained with *E. coli* strain HMS174 pJM9117, where a culture induced by heat shift at an optical density of approximately 1.0 yielded the highest concentration of PHB. The cells were grown in minimal media and the doubling time of the culture was considerable longer. Thus, the growth rate of the culture may be a significant factor in PHB production. In rapidly growing cultures, cell division is able to stay ahead of PHB production. Under these conditions, the cells do not produce enough PHB to inhibit growth. In slow growing cultures, cell division is not rapid enough to stay ahead of PHB production. Consequently, the cells accumulate PHB. Under slow growth conditions it would be better to allow the cell density to increase before initiating PHB production. During rapid growth, the best strategy is to induce PHB production early so that by the time the cells enter late log phase they contain adequate concentrations of the *phb* gene products and are able to rapidly shift to PHB production.

Example 18: Comparison of PHB production in *E. Coli* strain HMS174 pJM9238 grown in media with and without chloramphenicol

The purpose of this experiment was to compare PHB production in *E. coli* strain HMS174 pJM9238 in medium containing chloramphenicol and in medium without chloramphenicol. This is a measure of the stability of the plasmid without the selective pressure of the antibiotic under conditions in which PHB is produced. *E. coli* strain HMS174 pJM9238 was inoculated into 50 ml of LB medium containing 25 µg/ml chloramphenicol in a 250 ml baffled Erlenmeyer flask and incubated at 30°C, 175 rpm overnight. Two 1 liter baffled Erlenmeyer flasks containing 250 ml of LB broth + 2% glucose were prepared. Chloramphenicol was added to one flask to a final concentration of 25 µg/ml. The overnight culture was inoculated into each flask at a starting optical density at 600 nm of 0.10. The cultures were incubated at 38°C, 175 rpm in a Lab-Line Orbital Environ-Shaker (Lab-Line Instruments, Inc.). Multiple 3 ml samples were taken in duplicate over a 7 hour period to determine PHB production by GC analysis.

Results: PHB induction in each culture was nearly identical. At seven hours after induction, the culture containing chloramphenicol produced 1.65 mg/ml PHB, while the culture without chloramphenicol produced 1.58

mg/ml PHB (Figure 18). These results indicate that PHB can be produced efficiently in *E. coli* HMS174 pJM9238 in medium that does not contain chloramphenicol.

Example 19. Quantitation of PHB production in *tac* promoter *lac* Shine-Dalgarno-phbC fusion clones

E. coli strains HMS174 pJM9375 pMS421 and HMS174 pJM9376 pMS421 were tested for PHB production in liquid media. The cultures were inoculated into 3 ml of LB media containing 200 μg/ml ampicillin and 10 μg/ml streptomycin in 16x100 mm culture tubes and grown to saturation in a Lab-Line Incubator-Shaker at 37°C with shaking at 200 rpm. The cultures were diluted to an optical density at 600 nm of 0.10 into 50 ml of LB media containing 200 μg/ml ampicillin + 10 μg/ml streptomycin + 2% glucose. At an optical density of 2.0, IPTG was added to the culture to a final concentration of 10 mM. Aliquots of the cultures were withdrawn during growth for measuring optical density, dry weight analysis, and to quantitate PHB production as described above.

Results: PHB started to accumulate 30 minutes after the addition of IPTG to *E. coli* strain HMS174 pJM9375 pMS421 and strain HMS174 pJM9376 pMS421. Six hours after the addition of IPTG, the PHB level in the culture approached 3 mg/ml, compared to approximately 1 mg/ml for strain HMS174 pJM9232 pMS421 (previously determined in Example 15) (Figure 19). These results indicate that the replacement of the *phb* Shine-Dalgarno sequence with the *lac* Shine-Dalgarno sequence resulted in a 3-fold increase in the rate of synthesis of PHB from the multicopy plasmid vector.

Example 20. *Klebsiella* plasmid stability

Strain Constructions: The bacterial strain used in this study was *Klebsiella aerogenes* strain KC2671 *hutC515 recA3011* Δ[*bla*]-2. Strain KC2671 was streaked onto an LB plate and strain KC2671 pMS421 was streaked onto an LB + 10 μg/ml streptomycin plate from frozen permanents. Single colonies were picked from the plates and patched onto the same medium. These were used as stock plates. Strain KC2671 was inoculated into 3 ml of LB and strain KC2671 pMS421 was inoculated into 3 ml LB + 10 μg/ml streptomycin

and grown to saturation. One ml of each culture was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of the same medium in which they were previously grown. The strains were grown at 30°C to an optical density at 600 nm of 0.7-0.8 and were made electrocompetent as follows (during the following steps the cells were kept ice cold): The cultures were decanted into 50 ml Falcon tubes and placed on ice for 15 minutes, then centrifuged at 3000 rpm for 15 minutes. The supernatant was decanted and the pellets were resuspended in 50 ml of ice cold 10% glycerol. The cultures were centrifuged as before. The supernatant was aspirated off, then the pellets were resuspended in 20 ml of 10% glycerol. The cells were centrifuged as before, then resuspended in 10 ml of 10% glycerol in a 15 ml Falcon tube. The cultures were centrifuged as before, then resuspended in 200 µl 10% glycerol. 40 µl of the cell suspension were pipetted into chilled microfuge tubes and stored at -70°C.

To the KC2671 electrocompetent cells, 1 µl of plasmid pJM9238 was added, and the mixture was pipetted into an ice cold cuvette. To the KC2671 pMS421 electrocompetent cells, 1 µl of plasmid pJM9232 was added and the mixture was pipetted into an ice cold cuvette. The plasmid DNA was introduced into the cells by electroporation as previously described. After electroporation the cells were resuspended in 3 ml of LB medium and incubated at 30°C for 90 minutes, then 1, 10, and 100 µl of each culture was spread onto the appropriate antibiotic plates: strain KC2671 pJM8238 was plated onto LB + 25 µg/ml chloramphenicol and strain KC2671 pMS421 pJM9232 was plated onto LB + 10 µg/ml streptomycin + 50 µg/ml kanamycin. The plates were incubated overnight at 30°C.

Six well-isolated colonies of each strain were picked and patched onto the following plates: strain KC2671 pMS421 pJM9232 was patched onto a) LB + 10 µg/ml streptomycin + 50 µg/ml kanamycin, and b) LB + 10 µg/ml streptomycin + 50 µg/ml kanamycin + 1% glucose + 5 mM IPTG. Both plates were incubated at 30°C overnight. Strain KC2671 pJM9238 was patched onto a) LB + 25 µg/ml chloramphenicol and incubated at 30°C, and b) LB + 25 µg/ml chloramphenicol + 1% glucose and incubated at 37°C.

The glucose-containing plates were inspected and the apparent best PHB producer of each strain was chosen. The strains were then picked from the plates that did not contain glucose (that is, the best-producing cultures were then grown under conditions in which they did not make PHB and were then picked) and inoculated into 50 ml of LB containing the appropriate antibiotics. The cultures were incubated for 6-8 hours at 30°C (until the cultures were in early

stationary phase), then frozen permanents of each strain were made as described above and stored at -70°C.

Plasmid Stability: Four 50 ml cultures in 250 ml Erlenmeyer flasks were started from 500 µl of frozen permanents as follows: Strain KC2671 pJMS421 pJM9232 was inoculated into a) LB medium and b) LB + 10 µg/ml streptomycin + 50 µg/ml kanamycin. Strain KC2671 pJM9238 was inoculated into a) LB medium and b) LB + 25 µg/ml chloramphenicol. The cultures were incubated at a temperature of 30°C in an Innova shaker incubator at 200 rpm. The next morning each culture was diluted into sterile 0.85% saline solution and the 10^{-6}, 10^{-7}, and 10^{-8} dilutions were spread onto LB plates.

For plasmid preps, 1.0 ml of KC2671 pMS421 pJM9232 culture was pipetted into 1.5 ml microfuge tubes and centrifuged. The pellet was resuspended in 150 µl of SET buffer and stored at -20°C. For strain KC2671 pJM9238, 45 ml of culture was centrifuged in a 50 ml Falcon tube, resuspended in 4 ml of P1 buffer (QIAGEN kit), and stored at -20°C. For the first two days, 0.5 ml of the overnight culture was inoculated into 50 ml of fresh medium and the culture was incubated as before. After the second day, 125 µl of the overnight culture was used as the inoculum. The following day, 50 colonies were picked from each of the four cultures that had been plated, then the selected colonies were patched onto a 50 grid pattern as follows: For strain KC2671 pMS421 pJM9232, colonies were patched onto a) LB + kan, b) LB + strep, c) LB + kan + strep + glucose + IPTG, and d) LB media. The plates were incubated at 30°C. For strain KC2671 pJM9238, colonies were patched onto a) LB + chlor (30°C), b) LB + chlor (37°C), and c) LB (30°C). The next AM, 125 µl of the overnight culture was inoculated into fresh medium, the culture was grown overnight, and plated as described above. After 100 generations, colonies were replica plated onto screening plates for counting.

Results: For strain KC2672 pMS421 pJM9232, after 100 generations 100% (100/100) of the isolates tested from the LB culture and 100% (102/102) of the isolates tested from the LB + 10 µg/ml streptomycin + 50 µg/ml kanamycin culture retained streptomycin and kanamycin resistance. All isolates also produced PHB on plates containing glucose. For strain KC2671 pJM9238, after 100 generations, 99.4% (310/312) of the isolates tested from the culture grown in LB + 25 µg/ml chloramphenicol retained chloramphenicol resistance and produced PHB on plates containing glucose. After 100 generations, 89.8% (212/236) of the isolates tested from the culture grown in LB retained chloramphenicol resistance and produced PHB on plates containing glucose.

The loss of plasmid pJM9238 was first observed after 44 generations. The kinetics of plasmid pJM9238 segregation during growth in nonselective media are depicted in Figure 20, panel a.

Based on the above, when KC2671 pMS421 pJM9232 and KC2671 pJM9238 were grown selectively (*i.e.*, in the presence of antibiotics) the *phb* genes were not deleted or mutated. This indicates that the presence of the *phb* operon does not inhibit cell growth. Surprisingly, when the strains were grown nonselectively (*i.e.*, without antibiotics), both the drug resistance markers and the *phb* genes were retained. Thus, pJM9232 and pJM9238 are stable in this strain, and are suitable for use in a large scale fermentation without the presence of antibiotics in the culture.

Example 21. *Klebsiella* steady-state *tac-phb* induction studies

Klebsiella strain KC2671 pJM9238 was grown to saturation overnight in 50 ml of LB + 25 µg/ml chloramphenicol in a 250 ml Erlenmeyer baffled flask at 30°C with shaking at 175 rpm. An aliquot of the culture was inoculated into 250 ml of LB media containing 2% glucose and 25 µg/ml chloramphenicol in a 1 liter Erlenmeyer baffled flask to yield an initial optical density at 600 nm of 0.10. The culture was incubated at a given temperature in the range of 30°C to 40°C with shaking at 175 rpm. The growth of the culture was followed by measuring the optical density at 600 nm. During exponential growth, samples of the culture were harvested for analysis of PHB production.

Results: PHB could be detected in all of the cultures 2 to 4 hours after inoculation. The results for two temperature induction experiments are shown in Figure 20, panel b. In the culture grown at 31°C, PHB production rose from 1.054 µg/ml (0.405% of dry weight) at 3.0 hours after inoculation to 234.4 µg/ml (19.2% of dry weight) at 6.7 hours, then to 716.7 µg/ml (33.8% of dry weight) at 24 hours. In the culture grown at 33°C, PHB production rose from 10.4 µg/ml (1.305% of dry weight) at 2.75 hours after inoculation to 758 µg/ml (32.5% of dry weight) at 5.8 hours, then to 5.416 mg/ml (55.7% of dry weight) at 24 hours. Increasing the temperature above 33°C did not significantly increase the rate of PHB production relative to production at 33°C, and resulted in significantly smaller cells (as observed under light microscopy) and decreased yields. This may be a consequence of incubating the bacteria at a temperature well above the optimal range. These results indicate that in the *Klebsiella* strain KC2671, the *tac::phb* operon is repressed at 31°C, but expressed at 33°C.

Example 22. KC2671 pJM9238 fermentation

Klebsiella aerogenes strain KC2671 pJM9238 was tested for PHB production during fed-batch fermentation. The fermentor used in this study was a B. Braun Type ES10 Biostat E 15 liter fermentor. The parameters were controlled using the Micro-MFCS computer control system (B. Braun Melsungen AG) with a Hyundai Super-386C computer. The strain was inoculated from a frozen permanent into 50 ml of LB medium containing 25 µg/ml chloramphenicol and grown at 31°C to saturation. Approximately 10 ml of this culture was then inoculated into each of two 1 liter Fernbach flasks containing 250 ml of LB + 25 µg/ml chloramphenicol to obtain an initial optical density at 600 nm of 0.10. The cultures were incubated at 31°C, 175 rpm. At an optical density at 600 nm of approximately 3.5, the cultures were inoculated into 5 liters of media containing the following components: 6 g/L Na_2HPO_4 anhydrous, 6 g/L KH_2PO_4 anhydrous, 5 g/L $(NH_4)_2SO_4$, 0.35 g/L $MgSO_4 \cdot 7H_2O$, 3 ml/L trace elements, 5 g/L yeast extract. Chloramphenicol was added to the medium at a final concentration of 25 µg/ml. The feed media was composed of the following components: 33 g/L $(NH_4)_2SO_4$, 400 g/L glucose, 7 g/L $MgSO_4 \cdot 7H_2O$, 5 ml/L trace elements, 5 g/L yeast extract. The culture was incubated at 31°C to an optical density at 600 nm of approximately 3.0, at which time the temperature was shifted to 33°C. Aliquots were harvested at approximately 1 hour intervals for determination of dry weight, PHB content, and glucose concentrations. PHB content and dry weight were determined as previously described in Example 11. Glucose was quantitated using the Sigma Diagnostics Glucose Assay Kit (Sigma), Procedure No. 635, p. 5, as previously described in Example 15.

Results: The results are depicted in Figure 21, panel a. PHB production was effectively repressed in KC2671 pJM9238 when the culture was grown at 31°C. Prior to thermal induction, PHB levels were at or below 0.0441 mg/ml. After the incubation temperature was increased to 33°C, PHB synthesis was rapidly induced. At the 12 hour time point (approximately 6 hours after the temperature shift to 33°C) the PHB concentration was 7.017 mg/ml, an increase of over 150-fold. At the 24 hour time point (approximately 18 hours after the temperature shift) the PHB concentration was 27.4 mg/ml, an increase of over 600-fold. The rate of PHB synthesis observed in strain KC2671 pJM9238 was significantly higher than that previously observed in strain KC2671 pJM9131, as

shown in Figure 21, panel b. For example, at the 20 hour time point, the KC2671 pJM9238 culture contained 23 mg/ml PHB, while KC2671 pJM9131 contained only 10 mg/ml PHB.

The present embodiments of the present invention are to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes that come within the range and equivalency of the claims are therefore intended to be embraced therein.

Claims

1. A nucleic acid vector construct comprising a) a promoter comprising a -35 region of a *trp* promoter operably linked to a -10 region of a *lac* promoter, and b) an operator region of the *lac* promoter, said promoter being operably linked to said operator region and to a *phb* operon.

2. The vector construct of claim 1 wherein said promoter is a *tac* promoter.

3. The vector construct of claim 1 wherein said *phb* operon is derived from *Alcaligenes eutrophus*.

4. The vector construct of claim 1 wherein said construct further comprises a consensus Shine-Dalgarno sequence operably linked to a *phbC* gene of said *phb* operon.

5. The vector construct of claim 4 wherein said consensus Shine-Dalgarno sequence is a *lac* Shine-Dalgarno sequence.

6. The vector construct of claim 1 wherein said vector construct further comprises a consensus Shine-Dalgarno sequence operably linked to said *phb* operon, said sequence replacing the native *phbC* Shine-Dalgarno sequence.

7. The vector construct of claim 1 wherein said construct further comprises a stabilization locus.

8. The vector construct of claim 7 wherein said stabilization locus is *parB*.

9. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics of pJM9227.

10. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics of pJM9229.

11. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics of pJM9230.

12. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics of pJM9231.

13. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics of pJM9232.

14. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics pJM9233.

15. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics pJM9234.

16. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics pJM9235.

17. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics pJM9236.

18. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics pJM9237.

19. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics of pJM9238.

20. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics of pJM9375.

21. The vector construct of claim 1 wherein said vector construct has all of the essential characteristics of pJM9376.

22. The vector construct of claim 2 wherein said *tac* promoter and said *phb* operon are separated by a leader having a *cis*-acting positive regulatory element.

23. A runaway replicon nucleic acid vector construct including an expressible *phb* operon.

24. The nucleic acid vector construct of claim 23 wherein said *phb* operon is operably linked to and positioned downstream from a) a promoter comprising a -35 region of a *trp* promoter operably linked to a -10 region of a *lac* promoter, and b) an operator region of the *lac* promoter, said promoter also being operably linked to said operator region.

25. The nucleic acid vector construct of claim 24 wherein said promoter is a *tac* promoter.

26. The nucleic acid vector construct of claim 23 wherein said *phb* operon is derived from *Alcaligenes eutrophus*.

27. The vector construct of claim 23 wherein said runaway replicon vector construct includes a λ pR promoter operably linked to a *repA* gene.

28. The vector construct of claim 27 wherein the nucleic acid construct has all of the essential characteristics of pJM9117.

29. The vector construct of claim 23 or 24 wherein said construct further comprises a stabilization locus.

30. The vector construct of claim 29 wherein said stabilization locus in *parB*.

31. A nucleic acid construct having all of the essential characteristics of pJM9131.

32. A method for the production of poly-β-hydroxyalkanoate, comprising:

(a) introducing into a prokaryotic host cell a vector construct comprising a) a promoter comprising a -35 region of a *trp* promoter operably linked to a -10 region of a *lac* promoter, and b) an operator region of the *lac* promoter, said promoter being operably linked to said operator region and to a *phb* operon;

(b) culturing said host cell in an appropriate medium;

(c) adding an inducer to said medium, said inducer being capable of activating said promoter; and

(d) further culturing said host cell for a time sufficient to produce poly-β-hydroxyalkanoate.

33. The method of claim 32 further comprising the step of isolating poly-β-hydroxyalkanoate from said cultured host cell.

34. The method of claim 32 wherein said poly-β-hydroxyalkanoate is poly-β-hydroxybutyrate.

35. A method for the production of poly-β-hydroxyalkanoate, comprising:

(a) introducing into a prokaryotic host cell a runaway replicon vector construct comprising an expressible *phb* operon and a λ pR promoter operably linked to a *repA* gene, and λcI857 gene;

(b) culturing said host cell in a medium;

(c) increasing the temperature of said host cell, thereby inducing said runaway replicon vector construct; and

(d) further culturing said host cell for a time sufficient to produce poly-β-hydroxyalkanoate.

36. The method of claim 35 wherein said expressable *phb* operon of said runaway replicon vector construct comprises (i) a promoter that is negatively regulated by a repressor molecule, (ii) an operator region capable of binding said repressor molecule, and (iii) said *phb* operon, wherein said promoter is operably linked to said operator region and to said *phb* operon.

37. The method of claim 35 further comprising the step of isolating poly-β-hydroxyalkanoate from said cultured host cell.

38. The method of claim 35 wherein said poly-β-hydroxyalkanoate is poly-β-hydroxybutyrate.

39. The method of claim 32 or 35 wherein said host cell is an Enterobacteriaceae host cell.

40. The method of claim 39 wherein said Enterobacteriaceae host cell is *E. coli*.

41. The method of claim 39 wherein said Enterobacteriaceae is *Klebsiella*.

42. The method of claim 41 wherein said Enterobacteriaceae is *Klebsiella aerogenes*.

43. The method of claim 41 or 42 wherein said temperature is increased to about 33°C.

44. The method of claim 32 wherein said inducer is IPTG and said promoter is a *tac* promoter.

45. The method of claim 32 wherein step (a) further comprises introducing a *lacIq* gene into said prokaryotic host cell.

46. The method of claim 32 or 35 wherein said *phb* operon is derived from *Alcaligenes eutrophus*.

47. The method of claim 35 further comprising, during step (b), determining whether said culture of said host cells is growing rapidly or slowly; and during step (c), increasing said temperature early in a log phase of a growth cycle of said culture when said culture is slow-growing, or increasing said temperature late in a log phase of a growth cycle of said culture when said culture is fast-growing.

48. The method of claim 35 wherein said host cell is *E. coli* and said increase in temperature is to at least 36°C.

49. The method of claim 35 wherein said host cell is *Klebsiella* and said temperature increase is to about 33°C.

50. The method of claim 32 or 35 wherein said culture medium does not include an antibiotic.

51. An Enterobacteriaceae host cell containing a vector construct according to any one of claims 1, 2, 3, 23, 24, 25 and 26.

52. The Enterobacteriaceae of claim 51 wherein said Enterobacteriaceae is an *E. coli*.

53. The Enterobacteriaceae of claim 51 wherein said Enterobacteriaceae is *Klebsiella*.

54. Poly-β-hydroxybutyrate produced according to the method of claim 34 or 38.

A

phb promoter and phbC Shine-Dalgamo (pJM9131, pJM9117)

TTGACAGCGCGTGCGTTGCAAGGCAACAATGGACTCAA...288 bp...AGAGAGACAATCAAATCATG
−35 −10 +1 putative SD Met

B

tac promoter and phbC Shine-Dalgamo (72 bp leader)(pJM9229, pJM9236)

TTGACAATTAATCATCGGCTCGTATAATGTGTGGAATT...58 bp...AGAGAGACAATCAATCATG
−35 −10 +1 putative SD Met

C

tac promoter and phbC Shine-Dalgamo (355 bp leader)(pJM9232, pJM9238)

TTGACAATTAATCATCGGCTCGTATATGTGTGGAATT...334 bp...AGAGAGACAATCAAATCATG
−35 −10 +1 putative SD Met

D

tac promoter and lac Shine-Dalgamo (pJM9375)

TTGACAATTAATCATCGGCTCGGCTCGTATAATGTGTGGAATT...20 bp...CACAGGAAACAGGATCCCGTG
−35 −10 +1 SD SD Met

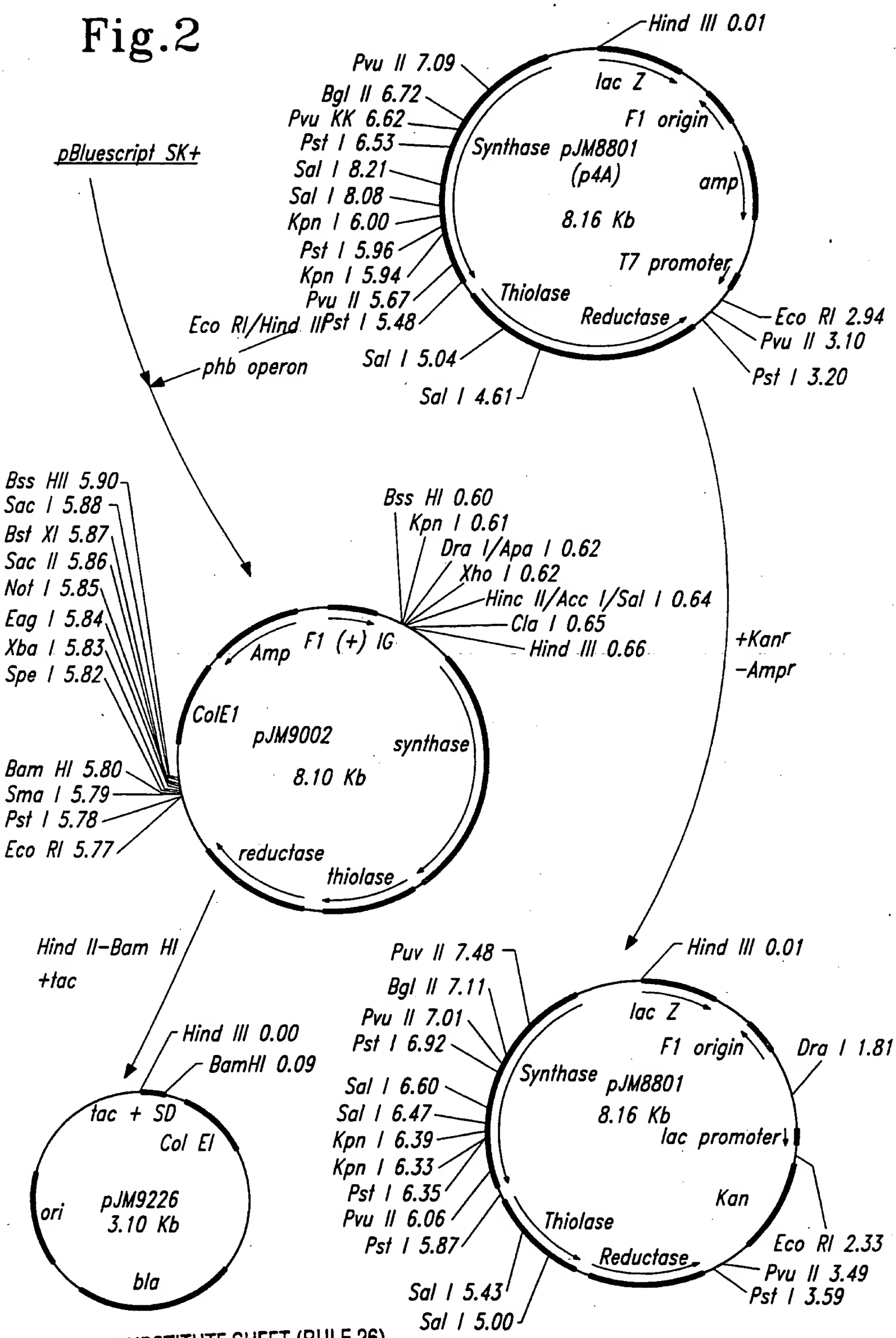
E

tac promoter and lac and phbC Shine-Dalgamo (pJM9376)

TTGACAATTAATCATCGGCTCGTATAATGTGFTGGAATT...20 bp..CACAGGACGGATCCCGAGACAATCAAATCATG
−35 −10 +1 SD SD putative SD Met

Fig. 1

Fig.2


pBluescript SK+
phb operon
Hind II–Bam HI
+tac
Hind III 0.01
lac Z
F1 origin
amp
T7 promoter
Eco RI 2.94
Pvu II 3.10
Pst I 3.20
Synthase pJM8801
(p4A)
8.16 Kb
Thiolase
Reductase
Pvu II 7.09
Bgl II 6.72
Pvu KK 6.62
Pst I 6.53
Sal I 8.21
Sal I 8.08
Kpn I 6.00
Pst I 5.96
Kpn I 5.94
Pvu II 5.67
Eco RI/Hind IIPst I 5.48
Sal I 5.04
Sal I 4.61
Bss HII 5.90
Sac I 5.88
Bst XI 5.87
Sac II 5.86
Not I 5.85
Eag I 5.84
Xba I 5.83
Spe I 5.82
Bam HI 5.80
Sma I 5.79
Pst I 5.78
Eco RI 5.77
Bss HI 0.60
Kpn I 0.61
Dra I/Apa I 0.62
Xho I 0.62
Hinc II/Acc I/Sal I 0.64
Cla I 0.65
Hind III 0.66
+Kanr
–Ampr
Amp
F1 (+) IG
ColE1
pJM9002
8.10 Kb
synthase
reductase
thiolase
Hind III 0.00
BamHI 0.09
tac + SD
Col EI
pJM9226
3.10 Kb
ori
bla
Puv II 7.48
Bgl II 7.11
Pvu II 7.01
Pst I 6.92
Sal I 6.60
Sal I 6.47
Kpn I 6.39
Kpn I 6.33
Pst I 6.35
Pvu II 6.06
Pst I 5.87
Sal I 5.43
Sal I 5.00
Hind III 0.01
lac Z
F1 origin
Dra I 1.81
Synthase pJM8801
8.16 Kb
lac promoter
Kan
Thiolase
Reductase
Eco RI 2.33
Pvu II 3.49
Pst I 3.59

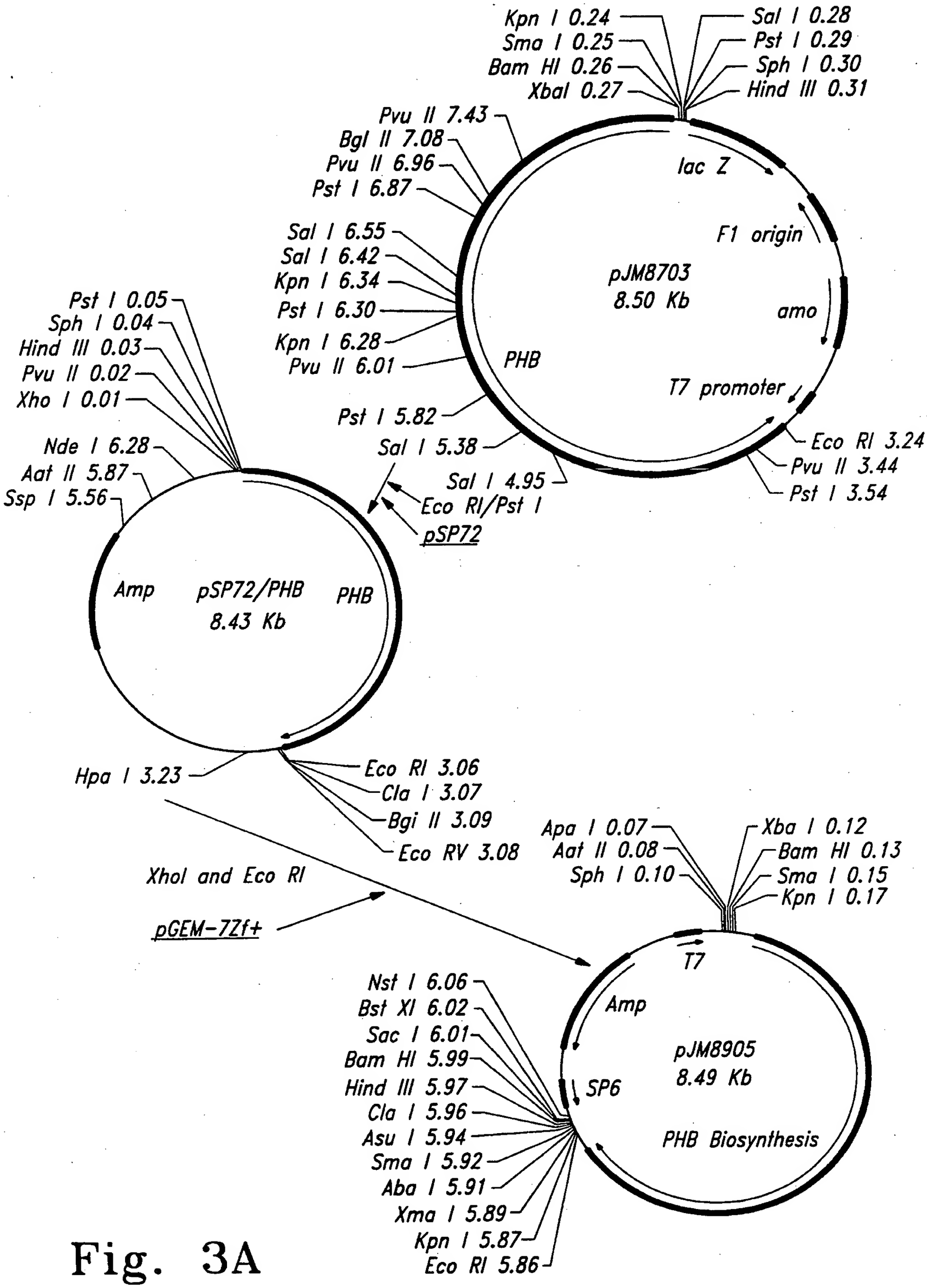

Kpn I 0.24
Sma I 0.25
Bam HI 0.26
Xbal 0.27
Sal I 0.28
Pst I 0.29
Sph I 0.30
Hind III 0.31
Pvu II 7.43
Bgl II 7.08
Pvu II 6.96
Pst I 6.87
Sal I 6.55
Sal I 6.42
Kpn I 6.34
Pst I 6.30
Kpn I 6.28
Pvu II 6.01
Pst I 5.82
Sal I 5.38
Sal I 4.95
lac Z
F1 origin
pJM8703
8.50 Kb
amo
PHB
T7 promoter
Eco RI 3.24
Pvu II 3.44
Pst I 3.54
Eco RI/Pst I
pSP72
Pst I 0.05
Sph I 0.04
Hind III 0.03
Pvu II 0.02
Xho I 0.01
Nde I 6.28
Aat II 5.87
Ssp I 5.56
Amp
pSP72/PHB
8.43 Kb
PHB
Hpa I 3.23
Eco RI 3.06
Cla I 3.07
Bgi II 3.09
Eco RV 3.08
XhoI and Eco RI
pGEM-7Zf+
Apa I 0.07
Aat II 0.08
Sph I 0.10
Xba I 0.12
Bam HI 0.13
Sma I 0.15
Kpn I 0.17
T7
Nst I 6.06
Bst XI 6.02
Sac I 6.01
Bam HI 5.99
Hind III 5.97
Cla I 5.96
Asu I 5.94
Sma I 5.92
Aba I 5.91
Xma I 5.89
Kpn I 5.87
Eco RI 5.86
Amp
SP6
pJM8905
8.49 Kb
PHB Biosynthesis

Fig. 3A

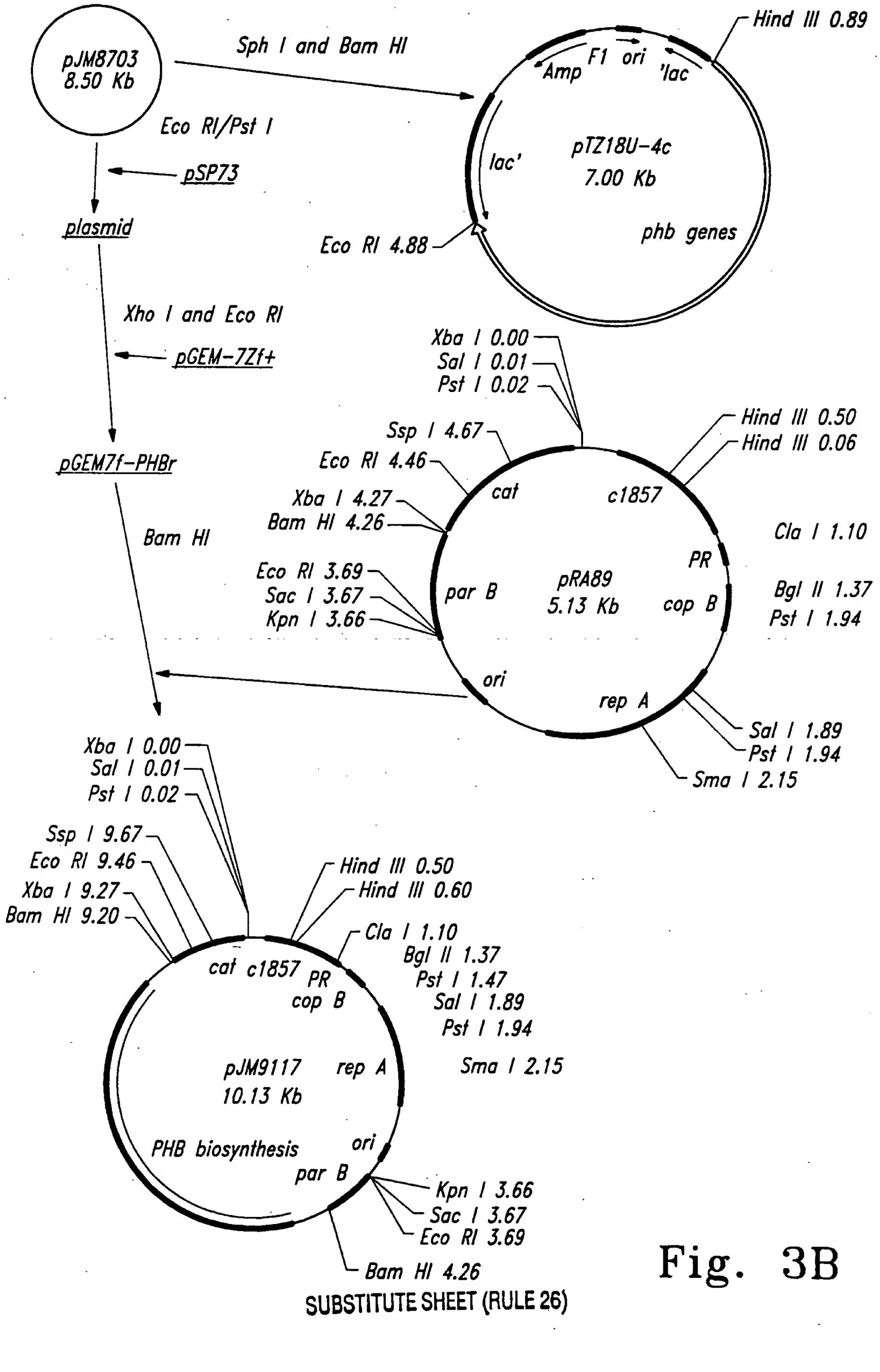

pJM8703
8.50 Kb
Sph I and Bam HI
Eco RI/Pst I
pSP73
plasmid
Xho I and Eco RI
pGEM-7Zf+
pGEM7f-PHBr
Bam HI
Hind III 0.89
Amp
F1 ori
'lac
lac'
pTZ18U-4c
7.00 Kb
phb genes
Eco RI 4.88
Xba I 0.00
Sal I 0.01
Pst I 0.02
Hind III 0.50
Hind III 0.06
Ssp I 4.67
Eco RI 4.46
Xba I 4.27
Bam HI 4.26
cat
c1857
Cla I 1.10
PR
Eco RI 3.69
Sac I 3.67
Kpn I 3.66
par B
pRA89
5.13 Kb
cop B
Bgl II 1.37
Pst I 1.94
ori
rep A
Sal I 1.89
Pst I 1.94
Sma I 2.15
Xba I 0.00
Sal I 0.01
Pst I 0.02
Ssp I 9.67
Eco RI 9.46
Xba I 9.27
Bam HI 9.20
Hind III 0.50
Hind III 0.60
Cla I 1.10
Bgl II 1.37
Pst I 1.47
Sal I 1.89
Pst I 1.94
Sma I 2.15
cat c1857
PR
cop B
pJM9117
10.13 Kb
rep A
PHB biosynthesis
ori
par B
Kpn I 3.66
Sac I 3.67
Eco RI 3.69
Bam HI 4.26

Fig. 3B

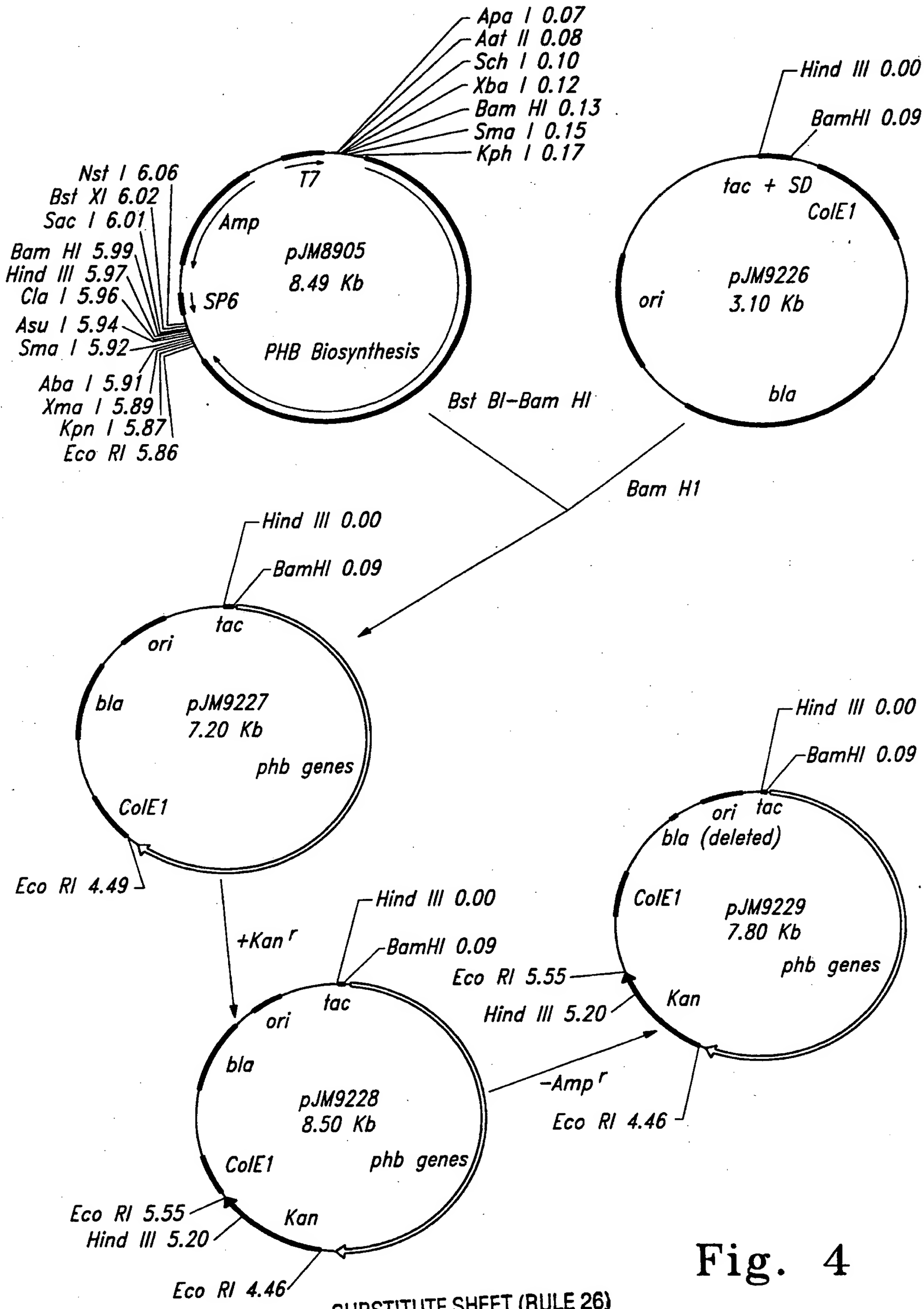

Apa I 0.07
Aat II 0.08
Sch I 0.10
Xba I 0.12
Bam HI 0.13
Sma I 0.15
Kph I 0.17
Nst I 6.06
Bst XI 6.02
Sac I 6.01
Bam HI 5.99
Hind III 5.97
Cla I 5.96
Asu I 5.94
Sma I 5.92
Aba I 5.91
Xma I 5.89
Kpn I 5.87
Eco RI 5.86
T7
Amp
pJM8905
8.49 Kb
SP6
PHB Biosynthesis
Hind III 0.00
BamHI 0.09
tac + SD
ColE1
pJM9226
3.10 Kb
ori
bla
Bst BI–Bam HI
Bam H1
Hind III 0.00
BamHI 0.09
tac
ori
bla
pJM9227
7.20 Kb
phb genes
ColE1
Eco RI 4.49
Hind III 0.00
BamHI 0.09
ori tac
bla (deleted)
ColE1
pJM9229
7.80 Kb
phb genes
Kan
Eco RI 5.55
Hind III 5.20
Eco RI 4.46
+Kan r
–Amp r
tac
ori
bla
pJM9228
8.50 Kb
ColE1
phb genes
Kan
Eco RI 5.55
Hind III 5.20
Eco RI 4.46

Fig. 4

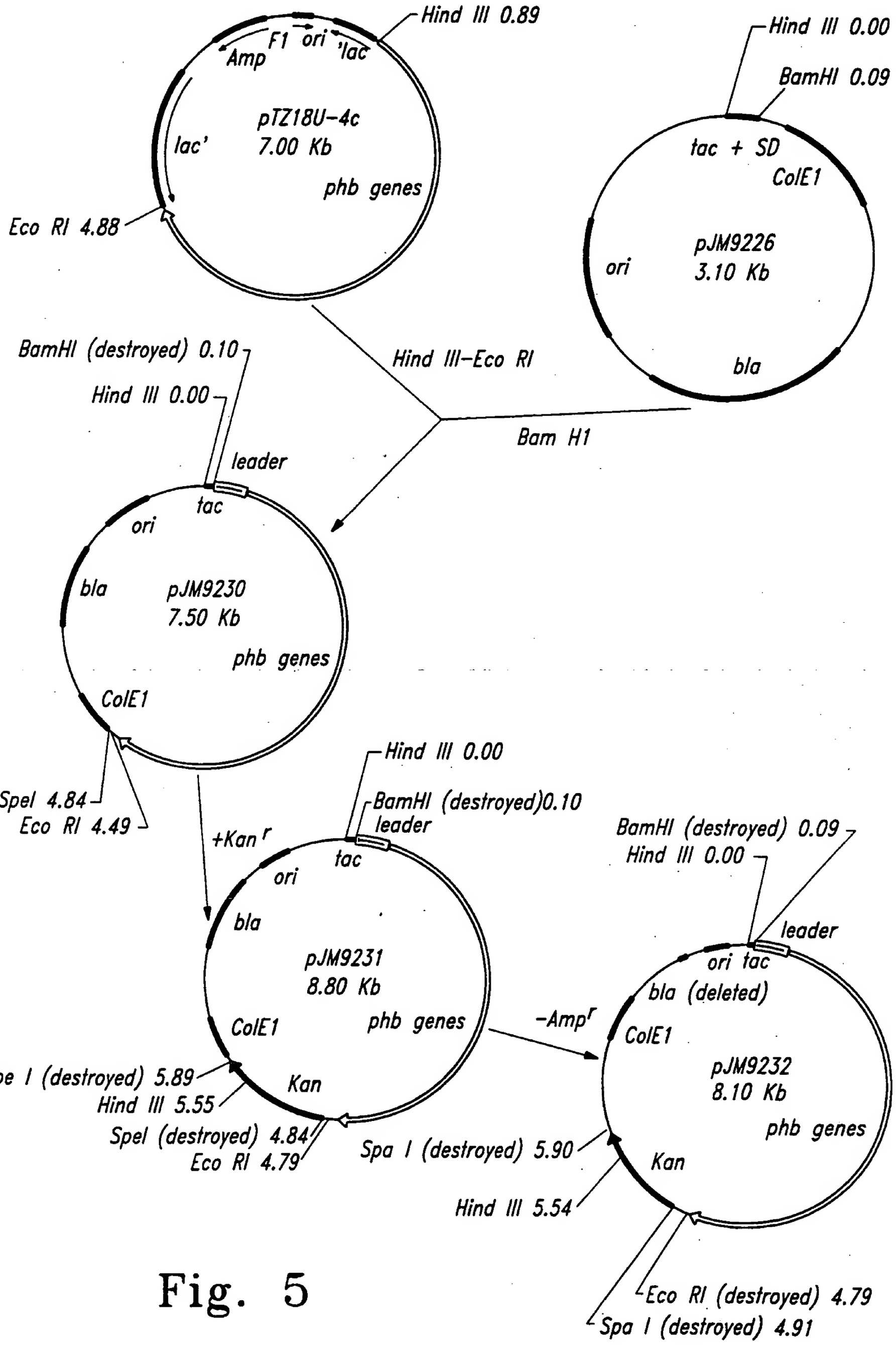

Hind III 0.89
F1 ori
Amp
'lac
pTZ18U-4c
7.00 Kb
lac'
phb genes
Eco RI 4.88
Hind III 0.00
BamHI 0.09
tac + SD
ColE1
pJM9226
3.10 Kb
ori
bla
Hind III–Eco RI
Bam H1
BamHI (destroyed) 0.10
Hind III 0.00
leader
tac
ori
bla
pJM9230
7.50 Kb
phb genes
ColE1
SpeI 4.84
Eco RI 4.49
+Kan r
Hind III 0.00
BamHI (destroyed)0.10
leader
tac
ori
bla
pJM9231
8.80 Kb
ColE1
phb genes
Spe I (destroyed) 5.89
Hind III 5.55
SpeI (destroyed) 4.84
Eco RI 4.79
Kan
BamHI (destroyed) 0.09
Hind III 0.00
leader
ori tac
bla (deleted)
-Amp r
ColE1
pJM9232
8.10 Kb
phb genes
Spa I (destroyed) 5.90
Hind III 5.54
Kan
Eco RI (destroyed) 4.79
Spa I (destroyed) 4.91

Fig. 5

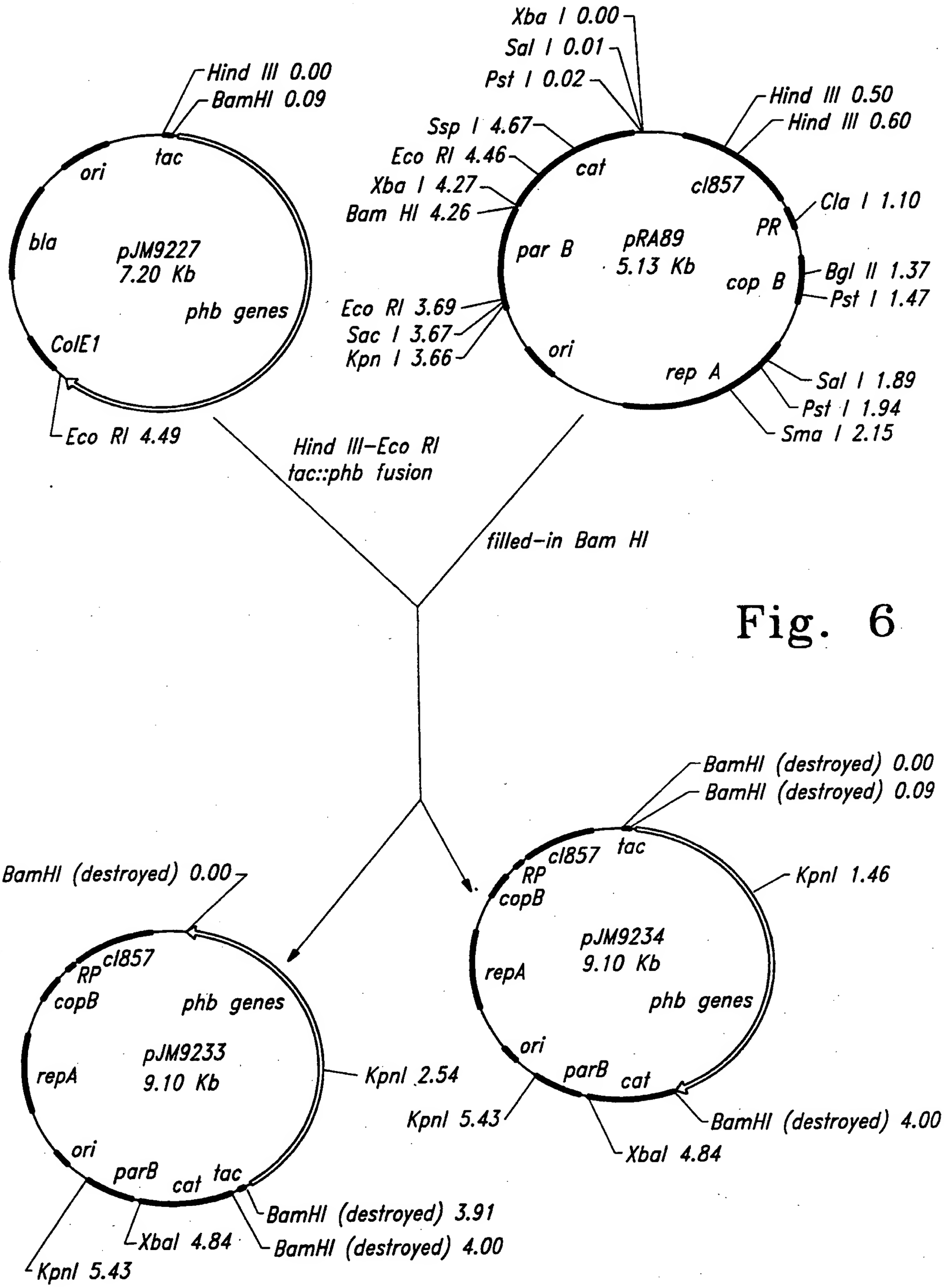

Hind III 0.00
BamHI 0.09
tac
ori
bla
pJM9227
7.20 Kb
phb genes
ColE1
Eco RI 4.49
Xba I 0.00
Sal I 0.01
Pst I 0.02
Hind III 0.50
Hind III 0.60
Ssp I 4.67
Eco RI 4.46
Xba I 4.27
Bam HI 4.26
cat
cI857
Cla I 1.10
PR
par B
pRA89
5.13 Kb
cop B
Bgl II 1.37
Pst I 1.47
Eco RI 3.69
Sac I 3.67
Kpn I 3.66
ori
rep A
Sal I 1.89
Pst I 1.94
Sma I 2.15
Hind III–Eco RI
tac::phb fusion
filled–in Bam HI
BamHI (destroyed) 0.00
BamHI (destroyed) 0.09
cI857
RP
copB
tac
KpnI 1.46
pJM9234
9.10 Kb
repA
phb genes
ori
parB
cat
BamHI (destroyed) 4.00
XbaI 4.84
KpnI 5.43
BamHI (destroyed) 0.00
cI857
RP
copB
phb genes
pJM9233
9.10 Kb
repA
KpnI 2.54
ori
parB
cat
tac
BamHI (destroyed) 3.91
BamHI (destroyed) 4.00
XbaI 4.84
KpnI 5.43

Fig. 6

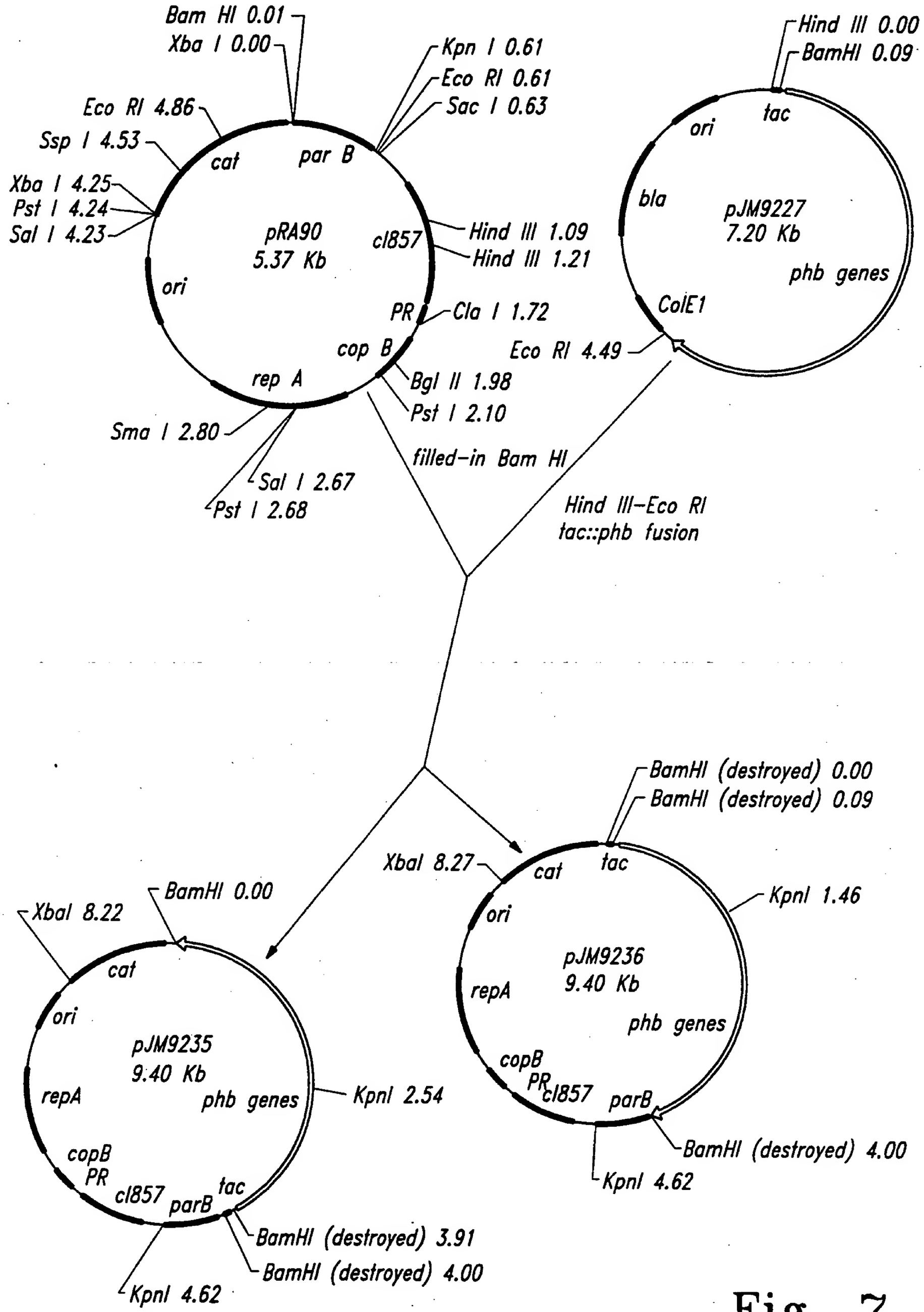

Bam HI 0.01
Xba I 0.00
Kpn I 0.61
Eco RI 0.61
Sac I 0.63
Eco RI 4.86
Ssp I 4.53
Xba I 4.25
Pst I 4.24
Sal I 4.23
cat
par B
pRA90
5.37 Kb
cI857
Hind III 1.09
Hind III 1.21
ori
PR
Cla I 1.72
cop B
rep A
Bgl II 1.98
Pst I 2.10
Sma I 2.80
Sal I 2.67
Pst I 2.68
filled-in Bam HI
Hind III-Eco RI
tac::phb fusion
Hind III 0.00
BamHI 0.09
ori
tac
bla
pJM9227
7.20 Kb
phb genes
ColE1
Eco RI 4.49
BamHI (destroyed) 0.00
BamHI (destroyed) 0.09
XbaI 8.27
cat
tac
ori
KpnI 1.46
pJM9236
9.40 Kb
repA
phb genes
copB
PR
cI857
parB
BamHI (destroyed) 4.00
KpnI 4.62
XbaI 8.22
BamHI 0.00
cat
ori
pJM9235
9:40 Kb
repA
phb genes
KpnI 2.54
copB
PR
cI857
parB
tac
BamHI (destroyed) 3.91
BamHI (destroyed) 4.00
KpnI 4.62

Fig. 7

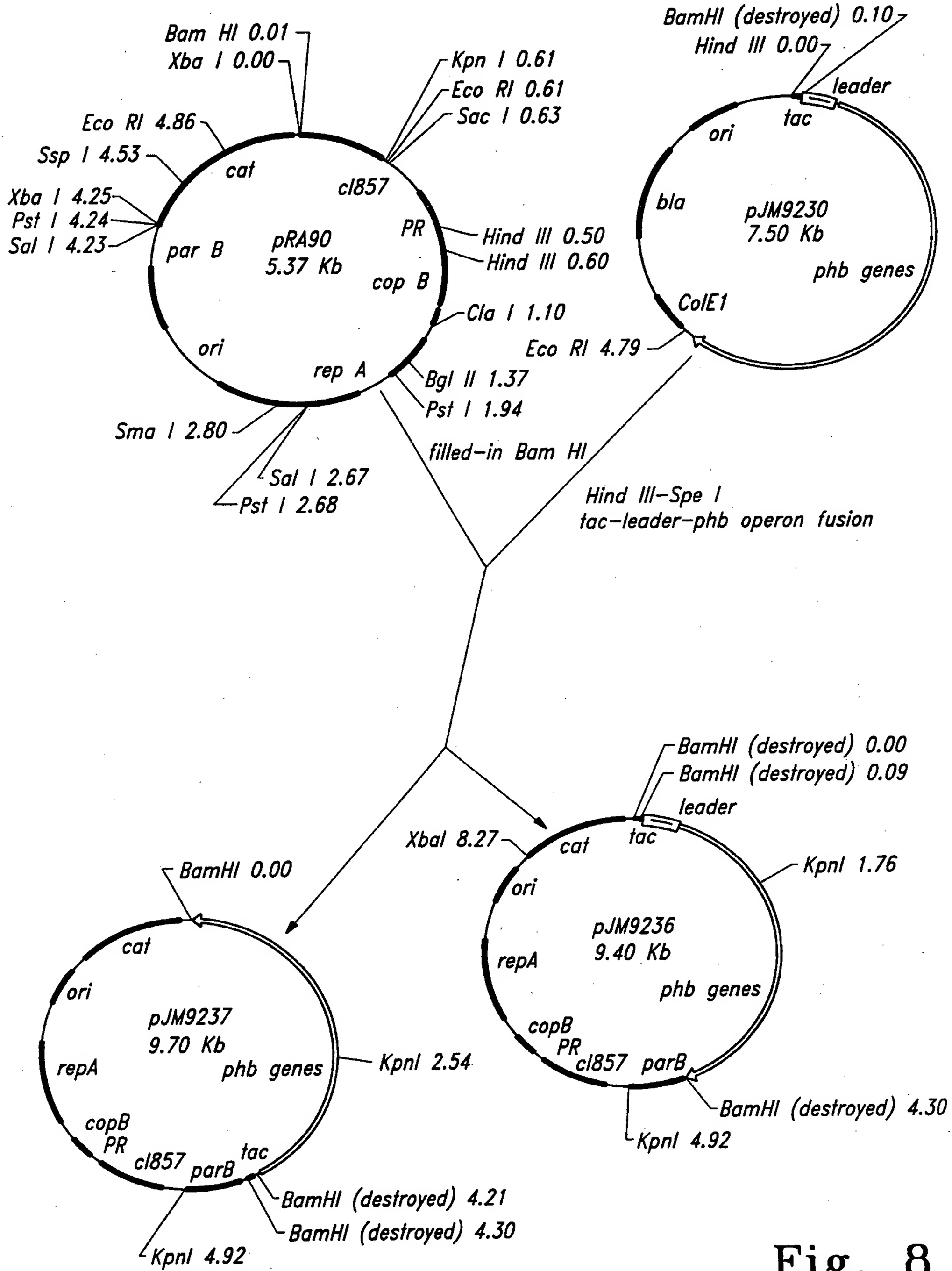

Bam HI 0.01
Xba I 0.00
Kpn I 0.61
Eco RI 0.61
Sac I 0.63
Eco RI 4.86
Ssp I 4.53
cat
cI857
Xba I 4.25
Pst I 4.24
Sal I 4.23
par B
pRA90
5.37 Kb
PR
Hind III 0.50
Hind III 0.60
cop B
Cla I 1.10
ori
rep A
Bgl II 1.37
Pst I 1.94
Sma I 2.80
Sal I 2.67
Pst I 2.68
filled-in Bam HI
Hind III-Spe I
tac-leader-phb operon fusion
BamHI (destroyed) 0.10
Hind III 0.00
leader
tac
ori
bla
pJM9230
7.50 Kb
phb genes
ColE1
Eco RI 4.79
BamHI (destroyed) 0.00
BamHI (destroyed) 0.09
leader
XbaI 8.27
cat
tac
KpnI 1.76
ori
pJM9236
9.40 Kb
repA
phb genes
copB
PR
cI857 parB
BamHI (destroyed) 4.30
KpnI 4.92
BamHI 0.00
cat
ori
pJM9237
9.70 Kb
repA
phb genes
KpnI 2.54
copB
PR
cI857
parB
tac
BamHI (destroyed) 4.21
BamHI (destroyed) 4.30
KpnI 4.92

Fig. 8

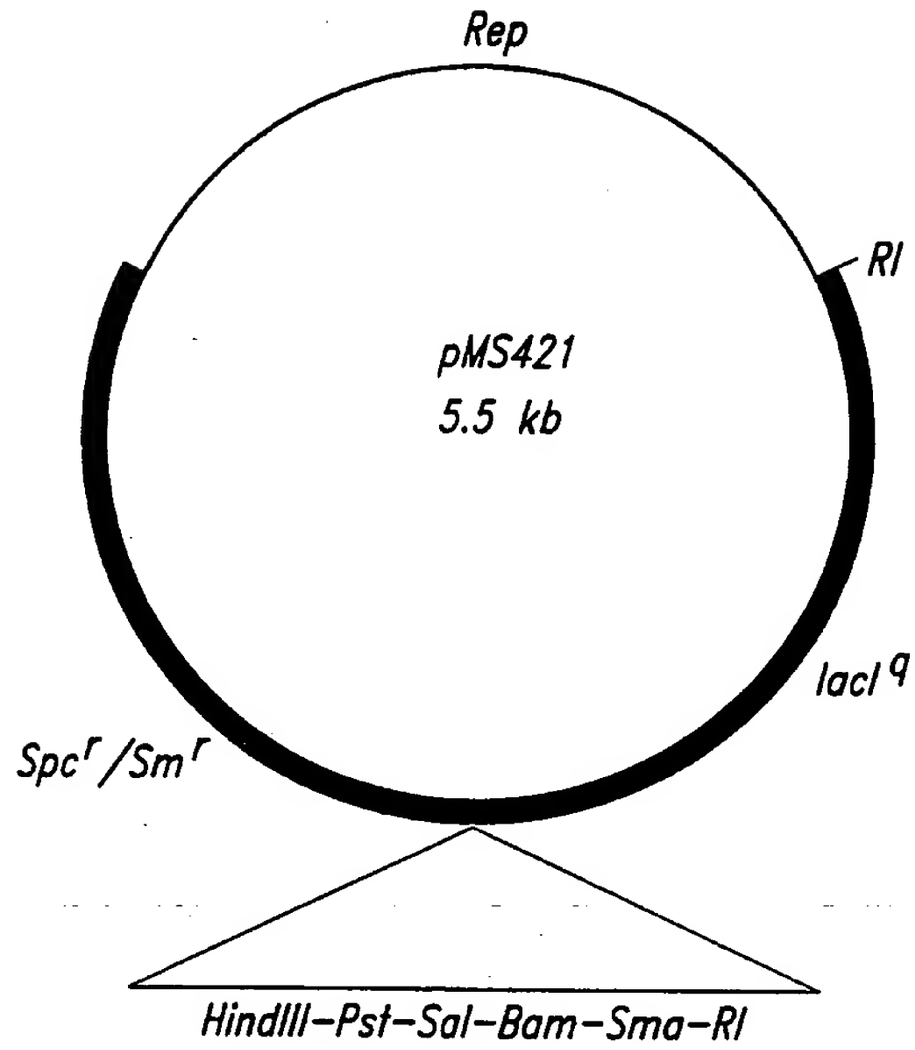


Fig.9

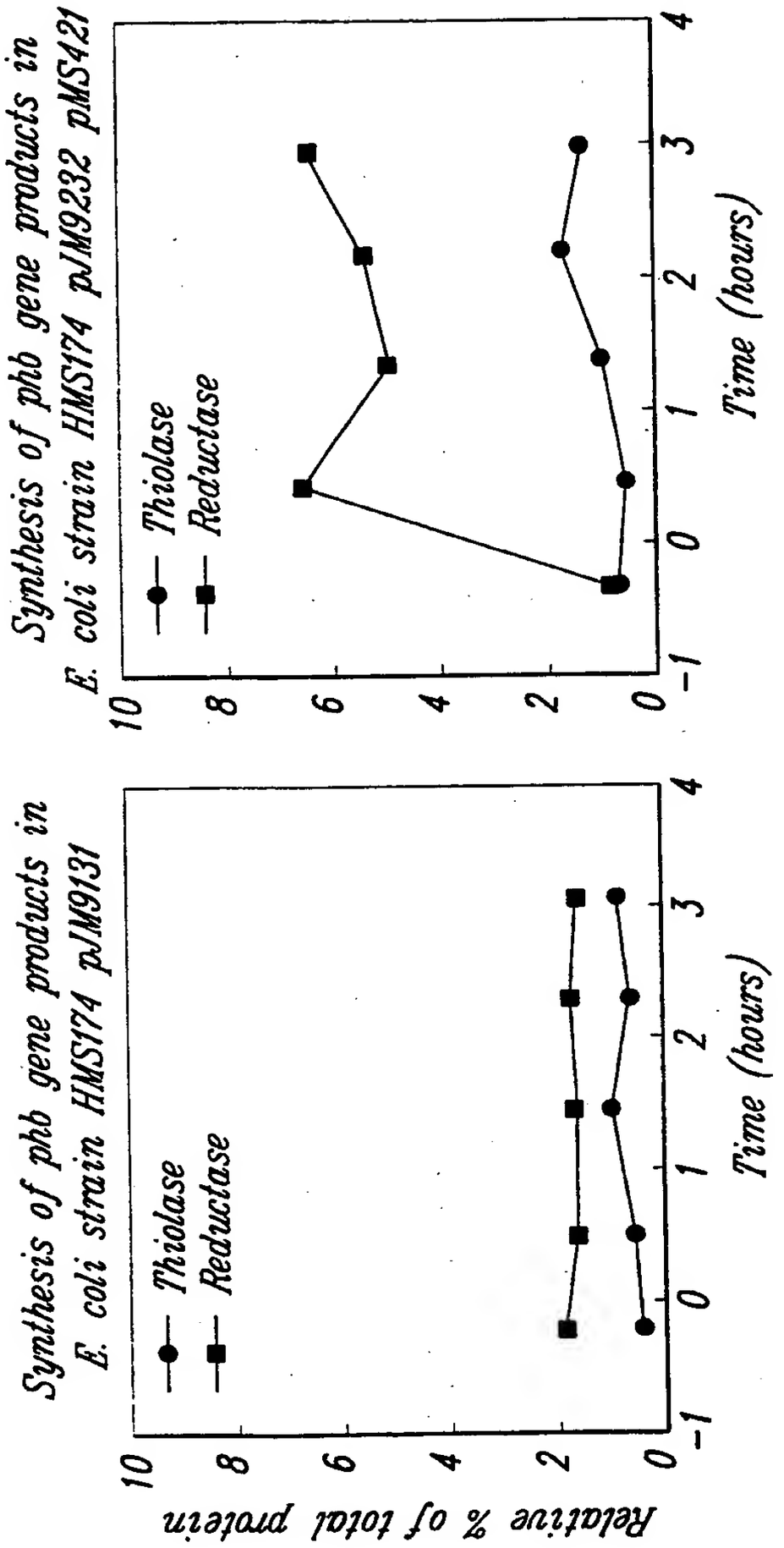


Fig. 10A

Fig. 10B

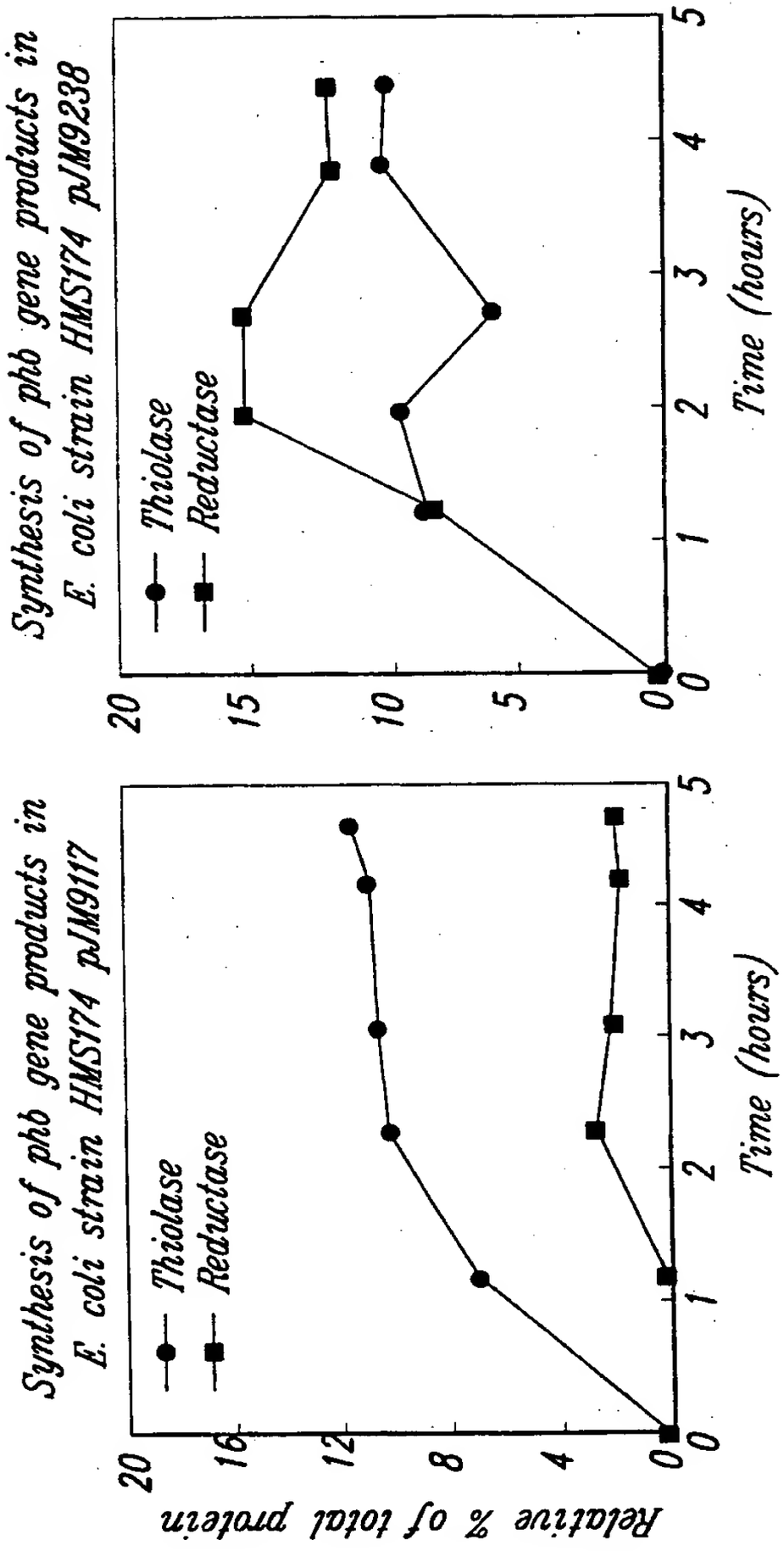


Fig. 10C

Fig. 10D

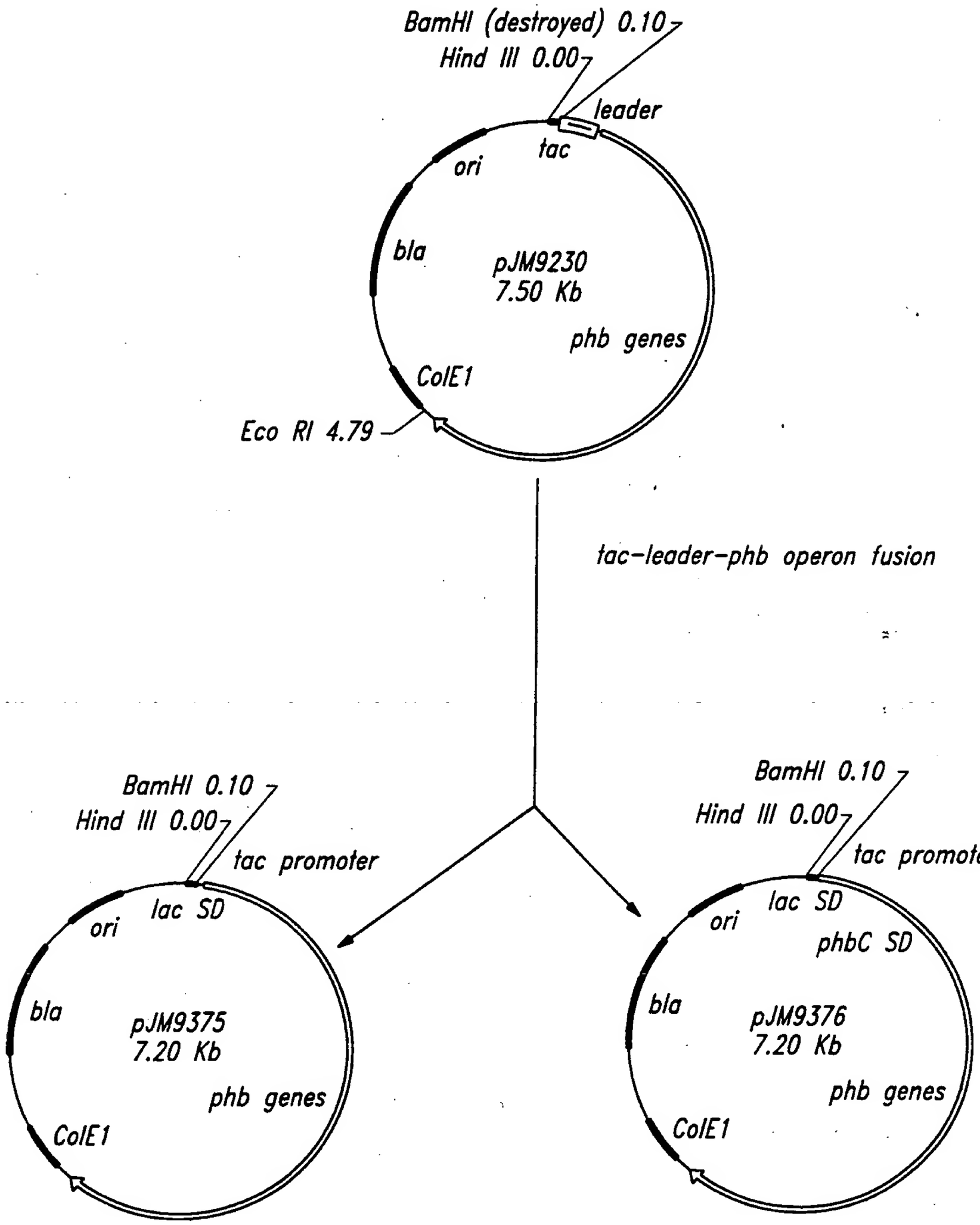

BamHI (destroyed) 0.10
Hind III 0.00
leader
tac
ori
bla
pJM9230
7.50 Kb
phb genes
ColE1
Eco RI 4.79
tac-leader-phb operon fusion
BamHI 0.10
Hind III 0.00
tac promoter
lac SD
ori
bla
pJM9375
7.20 Kb
phb genes
ColE1
BamHI 0.10
Hind III 0.00
tac promoter
lac SD
ori
phbC SD
bla
pJM9376
7.20 Kb
phb genes
ColE1

Fig.11

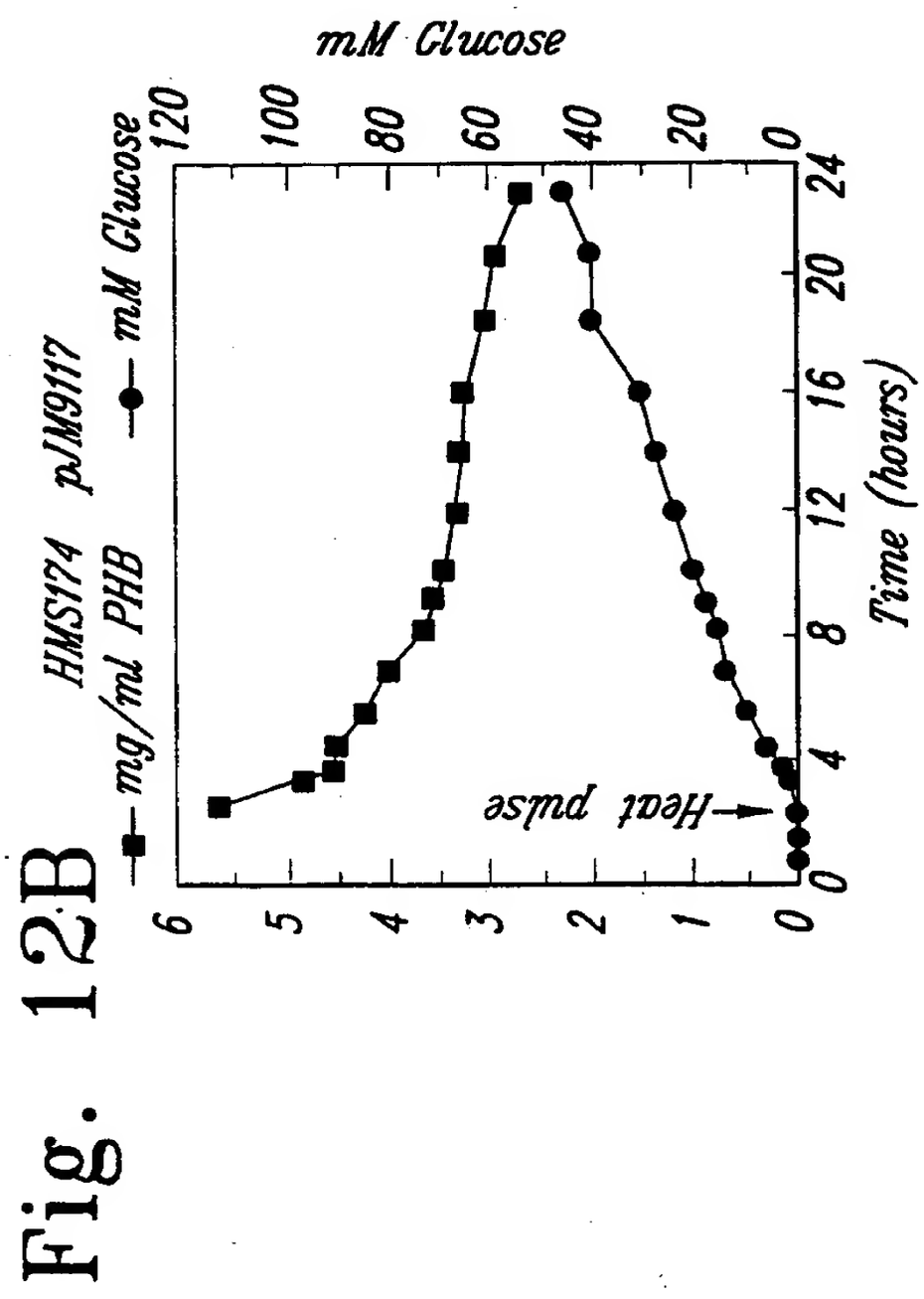


Fig. 12B

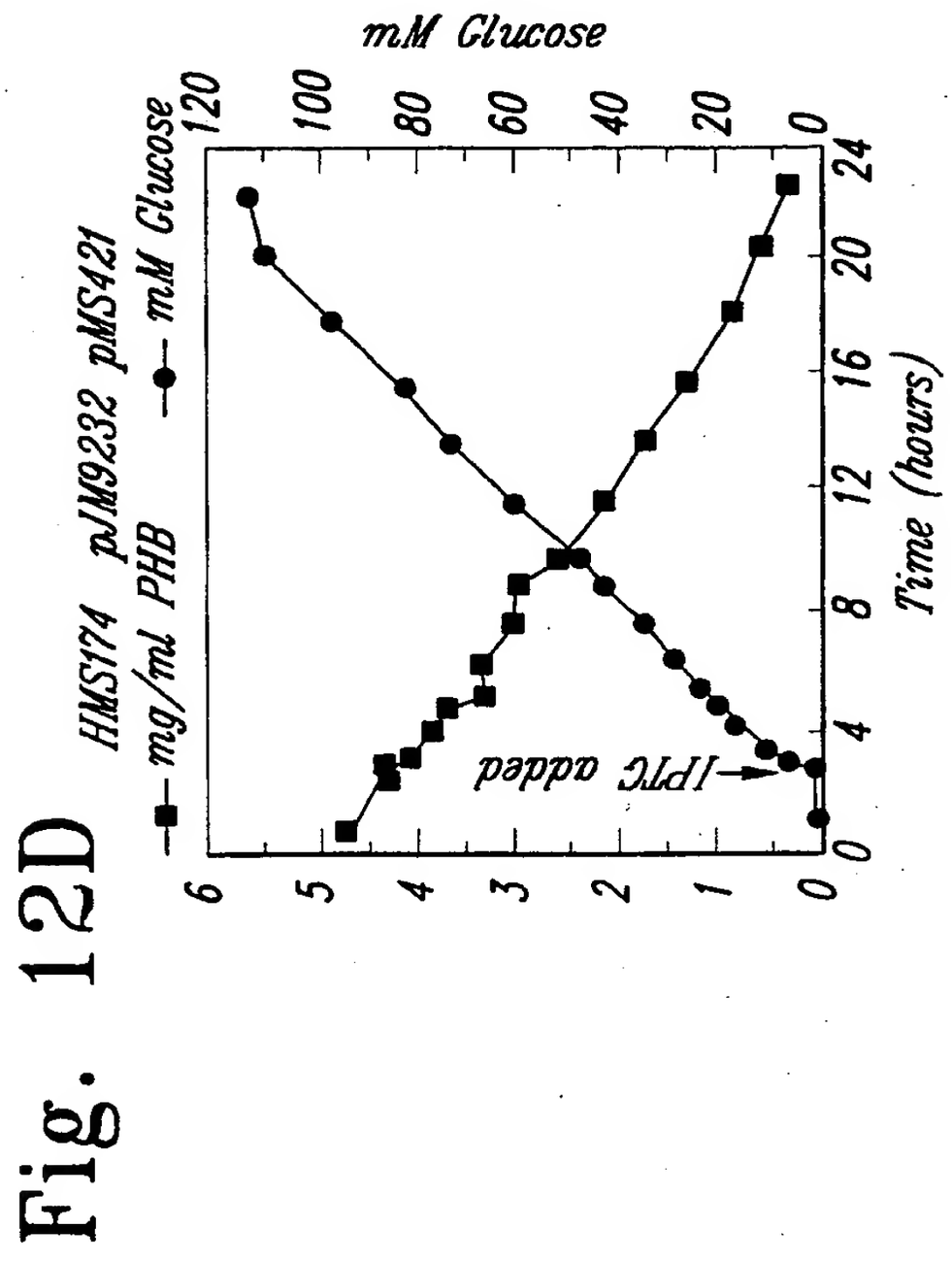


Fig. 12D

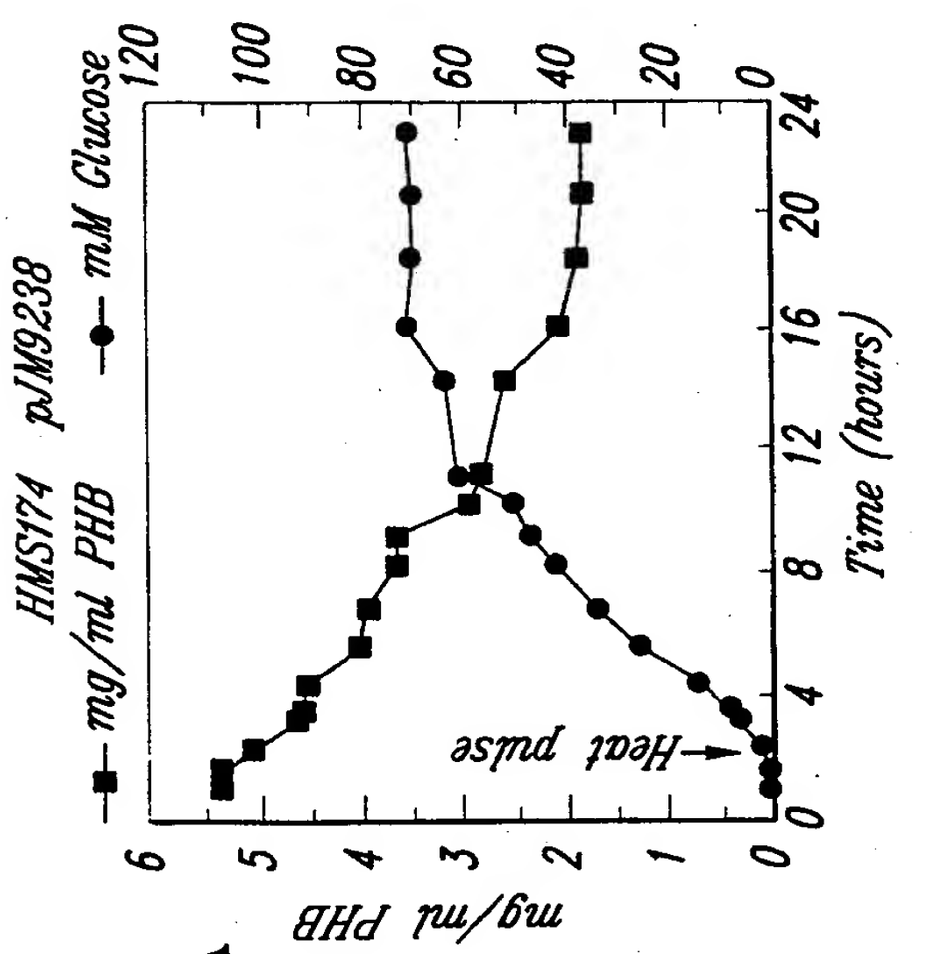


Fig. 12A

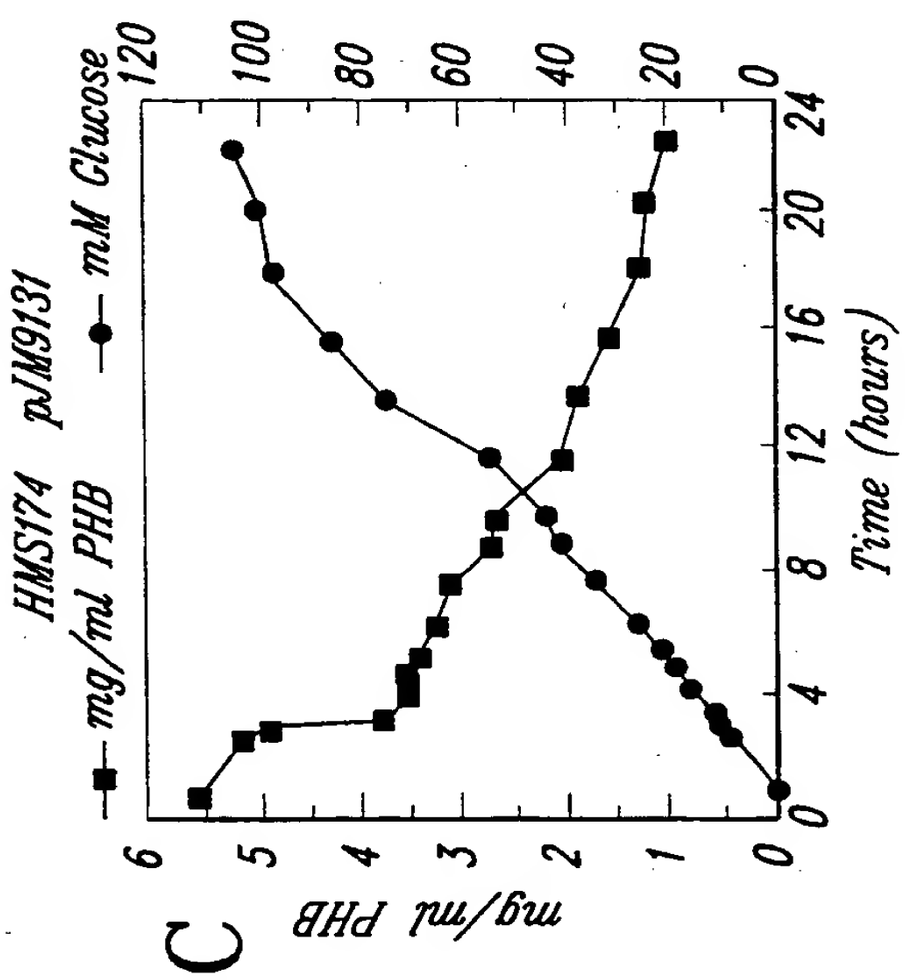


Fig. 12C

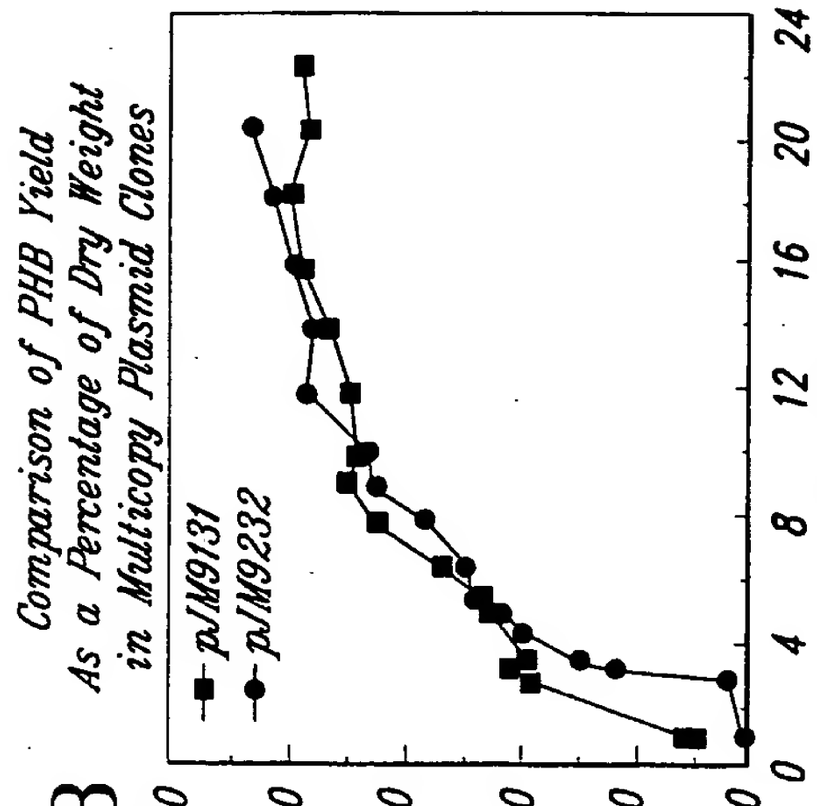


Fig. 13B

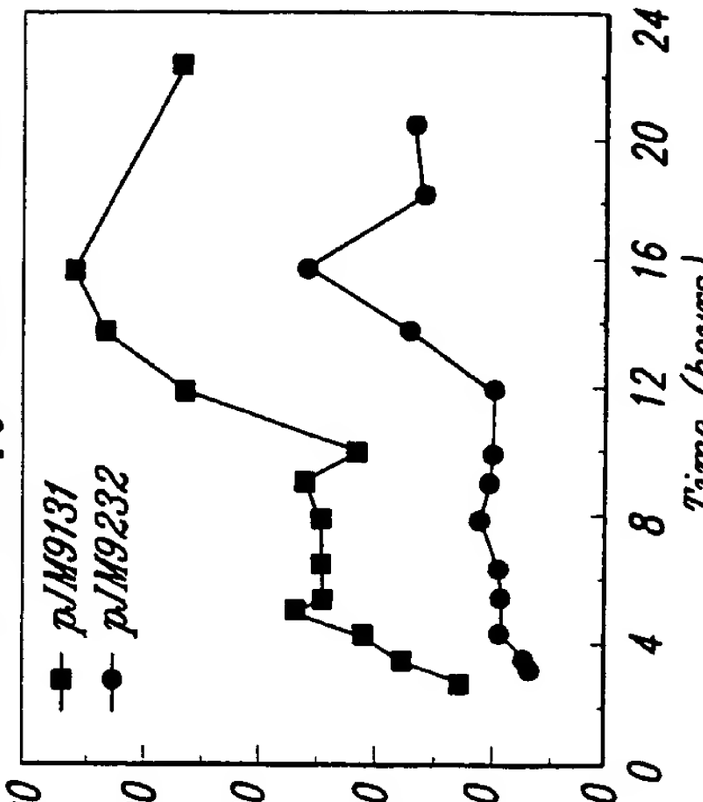


Fig. 14B

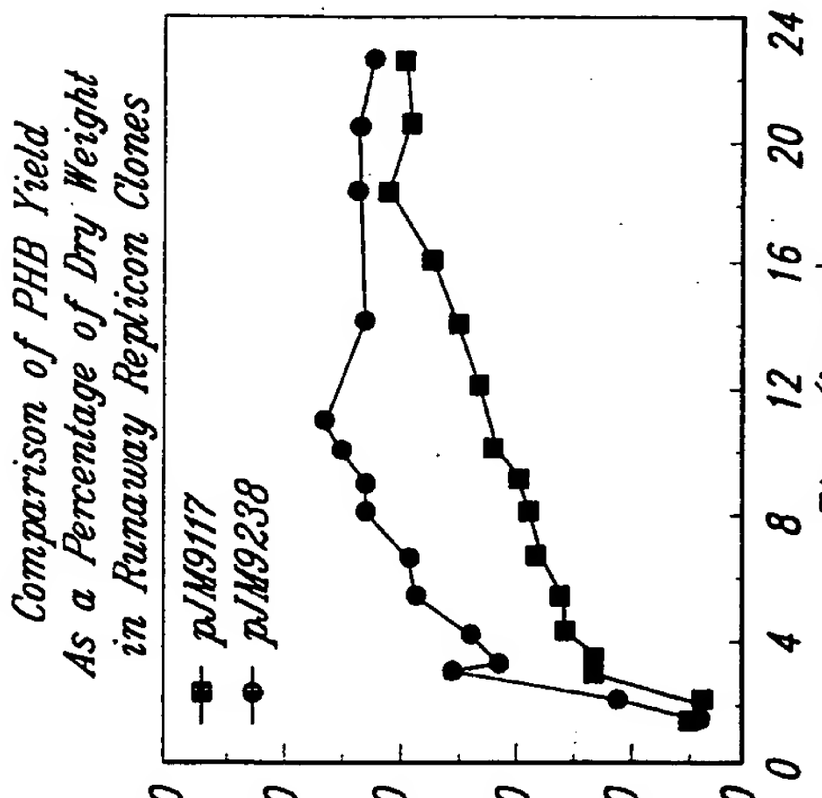


Fig. 13A

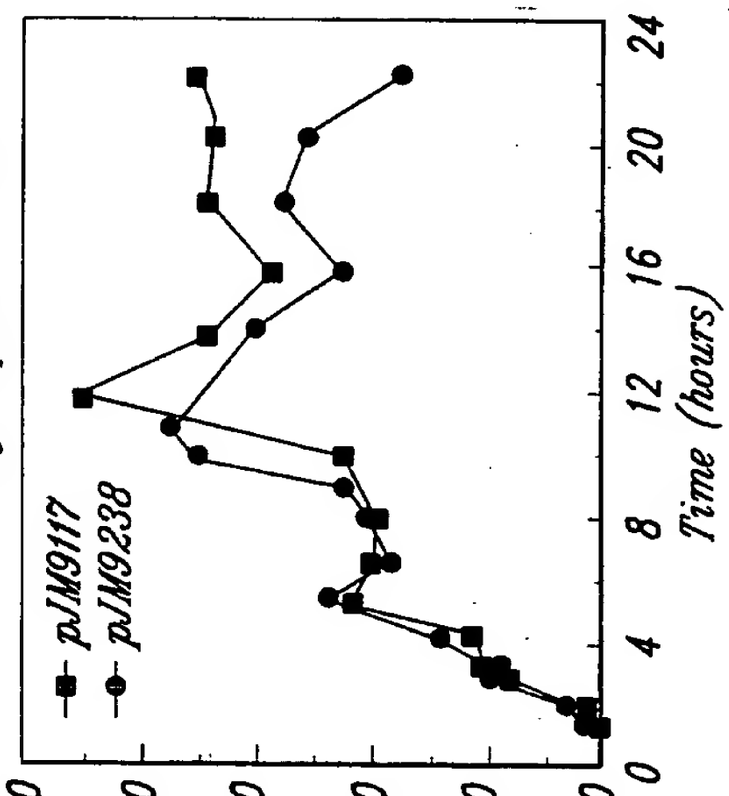


Fig. 14A

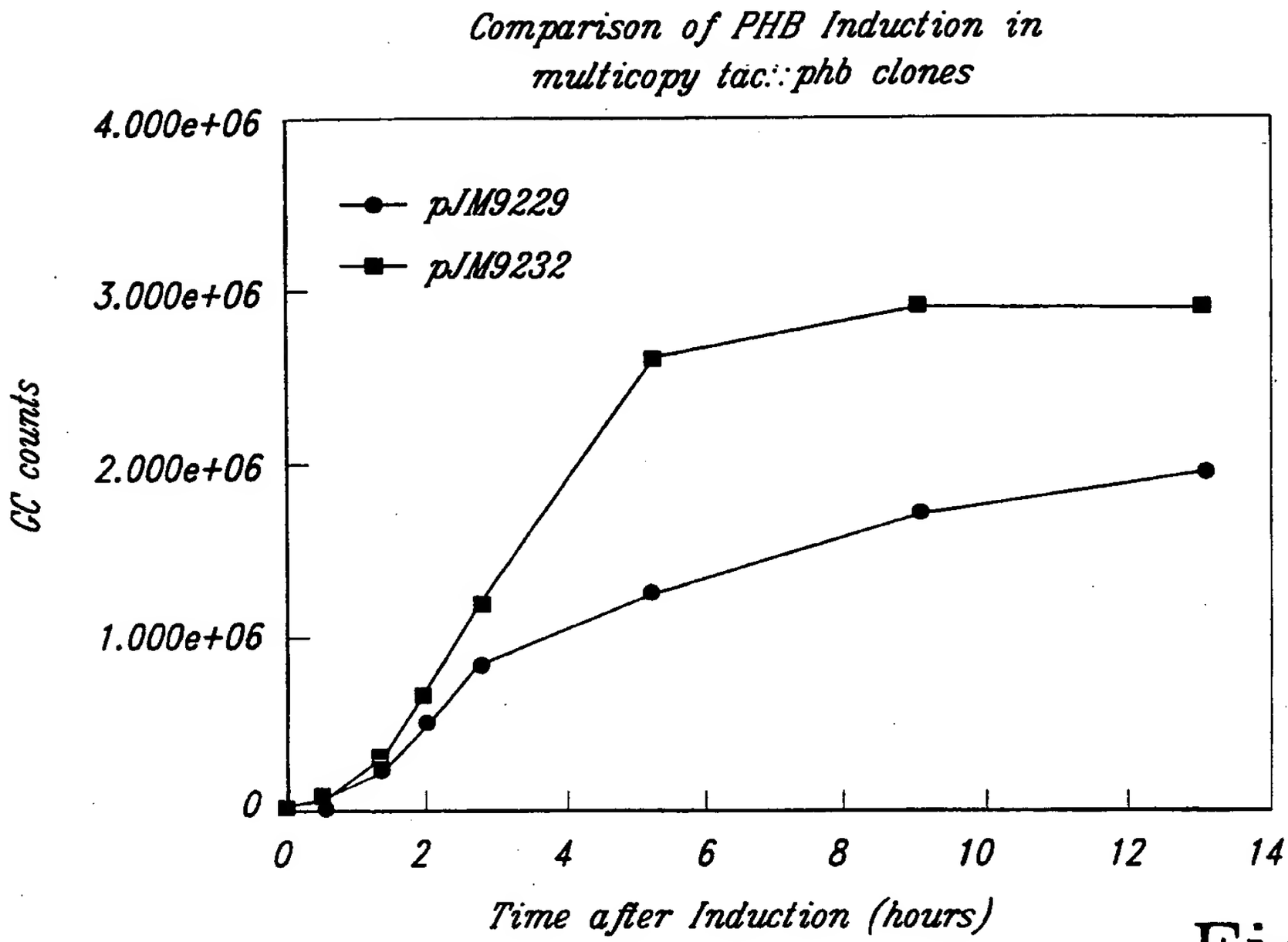

Comparison of PHB Induction in
multicopy tac::phb clones
4.000e+06
3.000e+06
2.000e+06
1.000e+06
0
CC counts
pJM9229
pJM9232
0
2
4
6
8
10
12
14
Time after Induction (hours)

Fig. 15

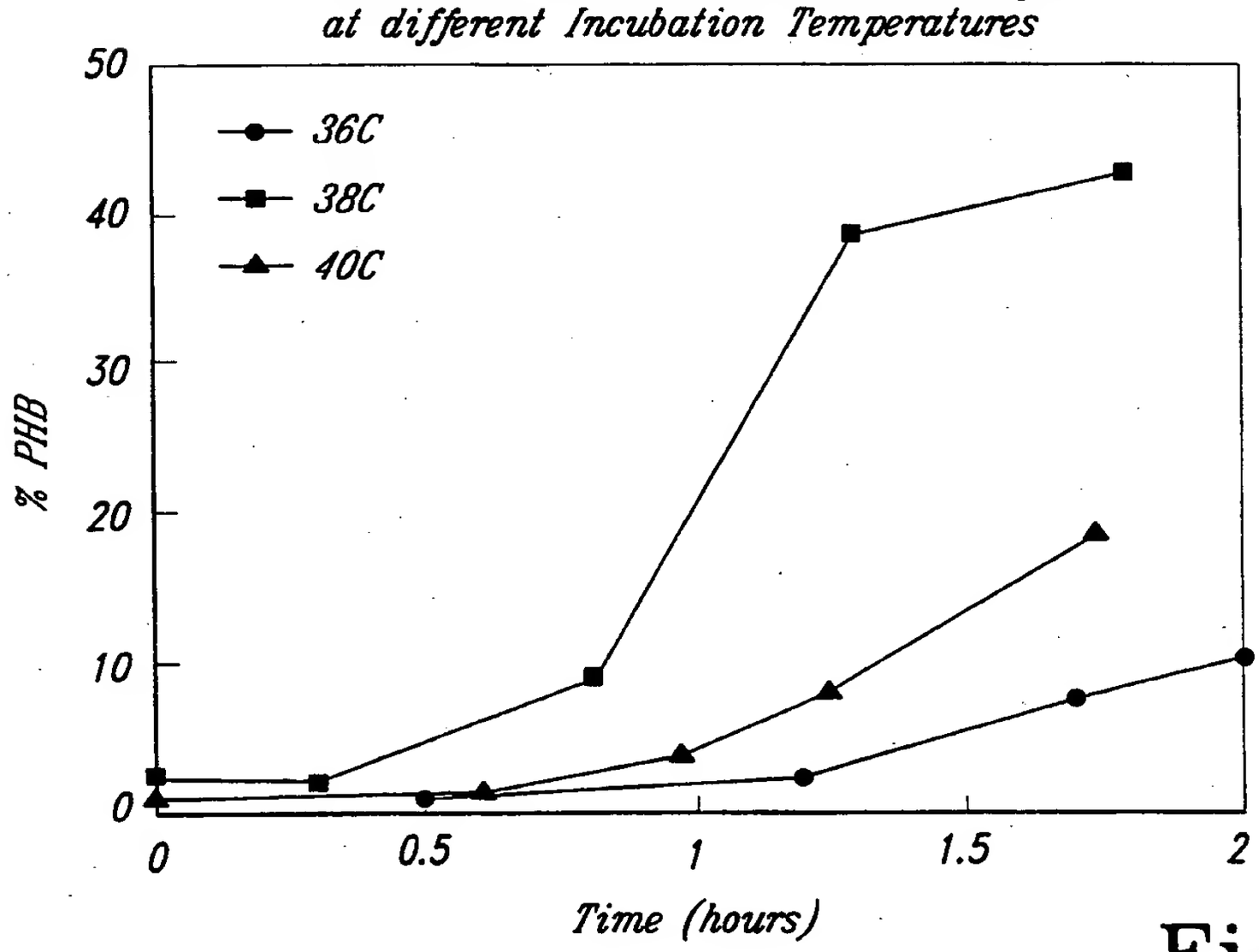

PHB Production in E.coli strain HMS174 pJM9238
at different Incubation Temperatures
50
40
30
20
10
0
% PHB
36C
38C
40C
0
0.5
1
1.5
2
Time (hours)

Fig. 16

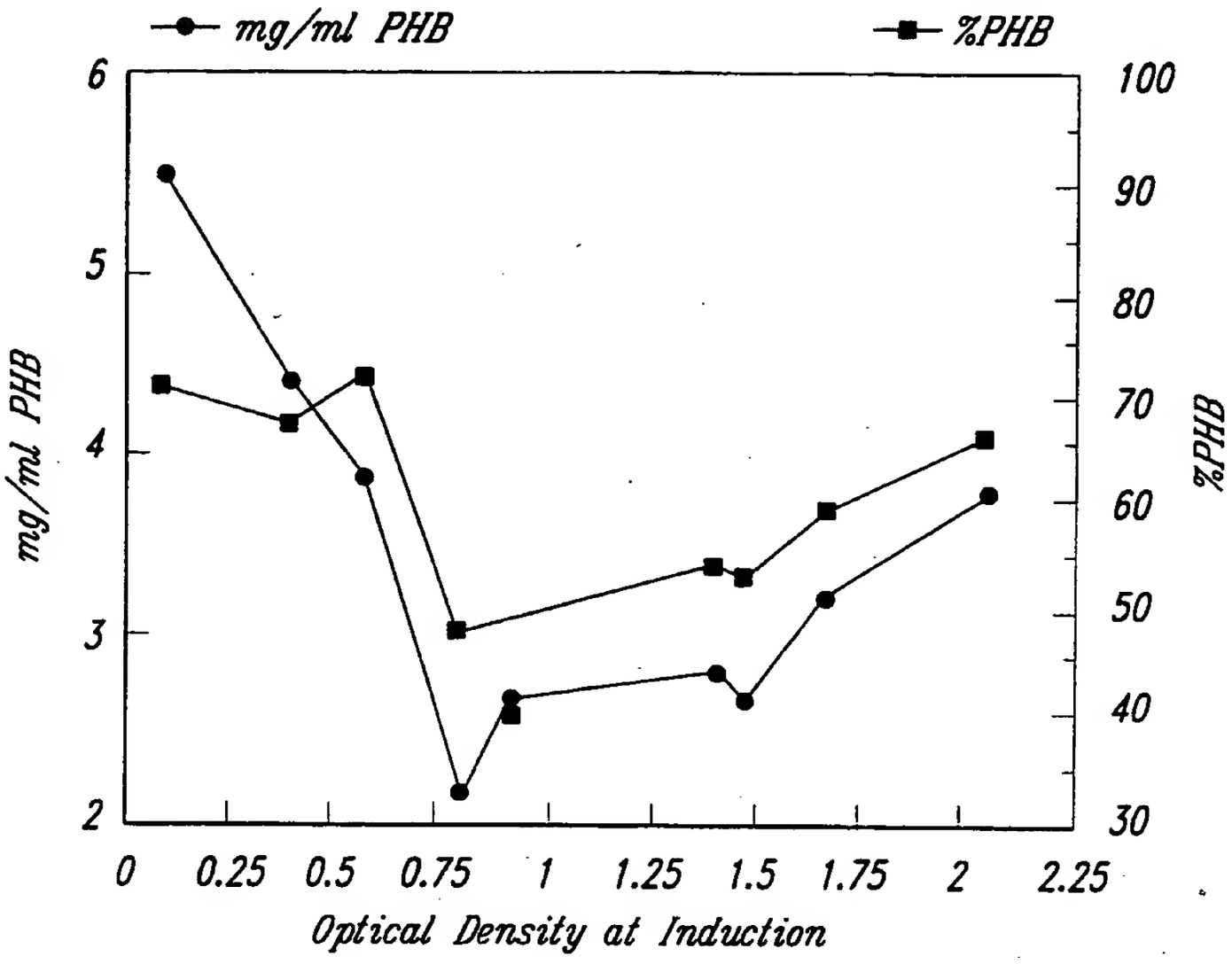

HMS174 pJM9238 Temp Shift Optimization
mg/ml PHB
%PHB
6
5
4
3
2
100
90
80
70
60
50
40
30
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
2.25
Optical Density at Induction

Fig. 17

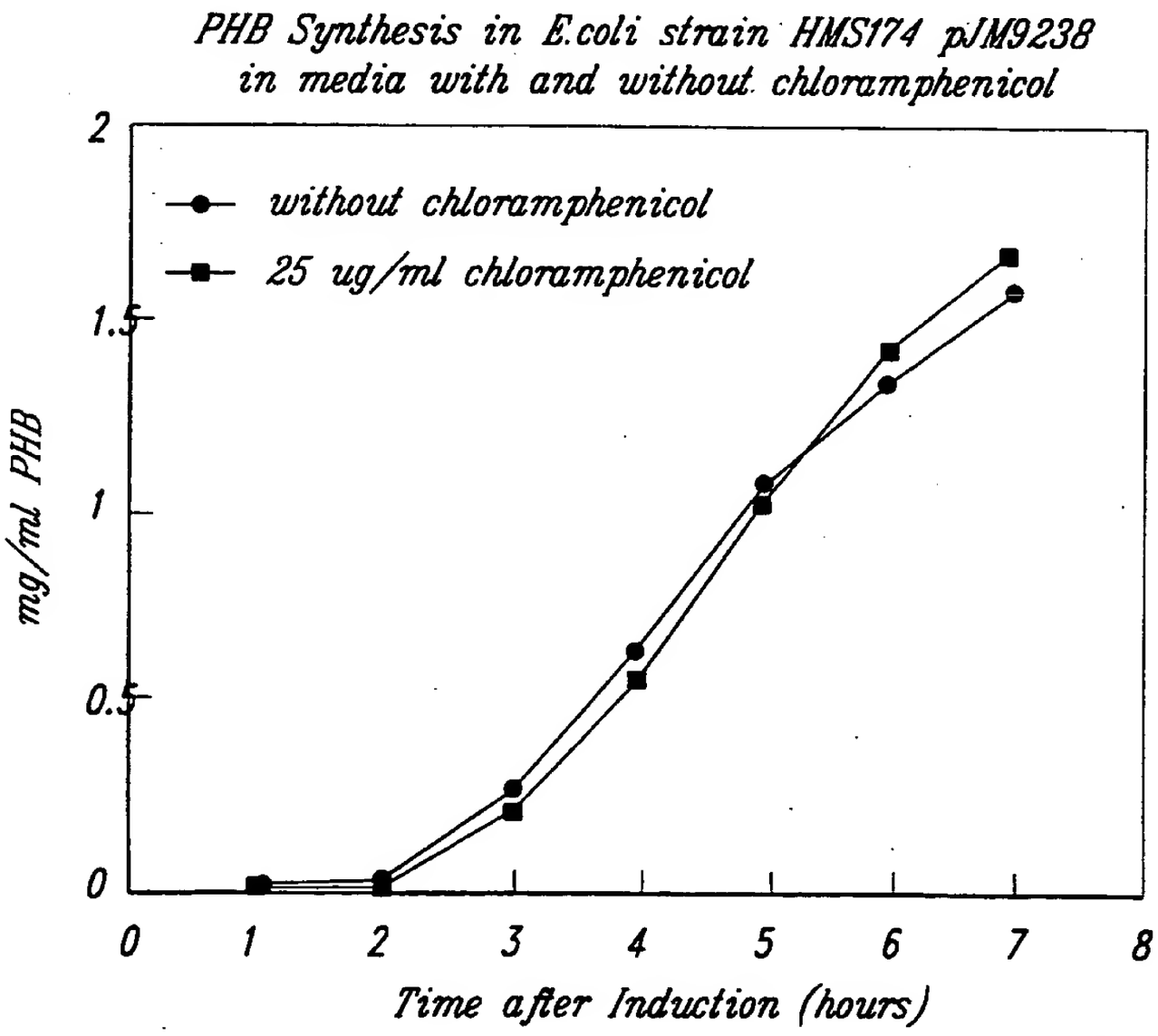

PHB Synthesis in E.coli strain HMS174 pJM9238
in media with and without chloramphenicol
without chloramphenicol
25 ug/ml chloramphenicol
mg/ml PHB
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
8
Time after Induction (hours)

Fig. 18

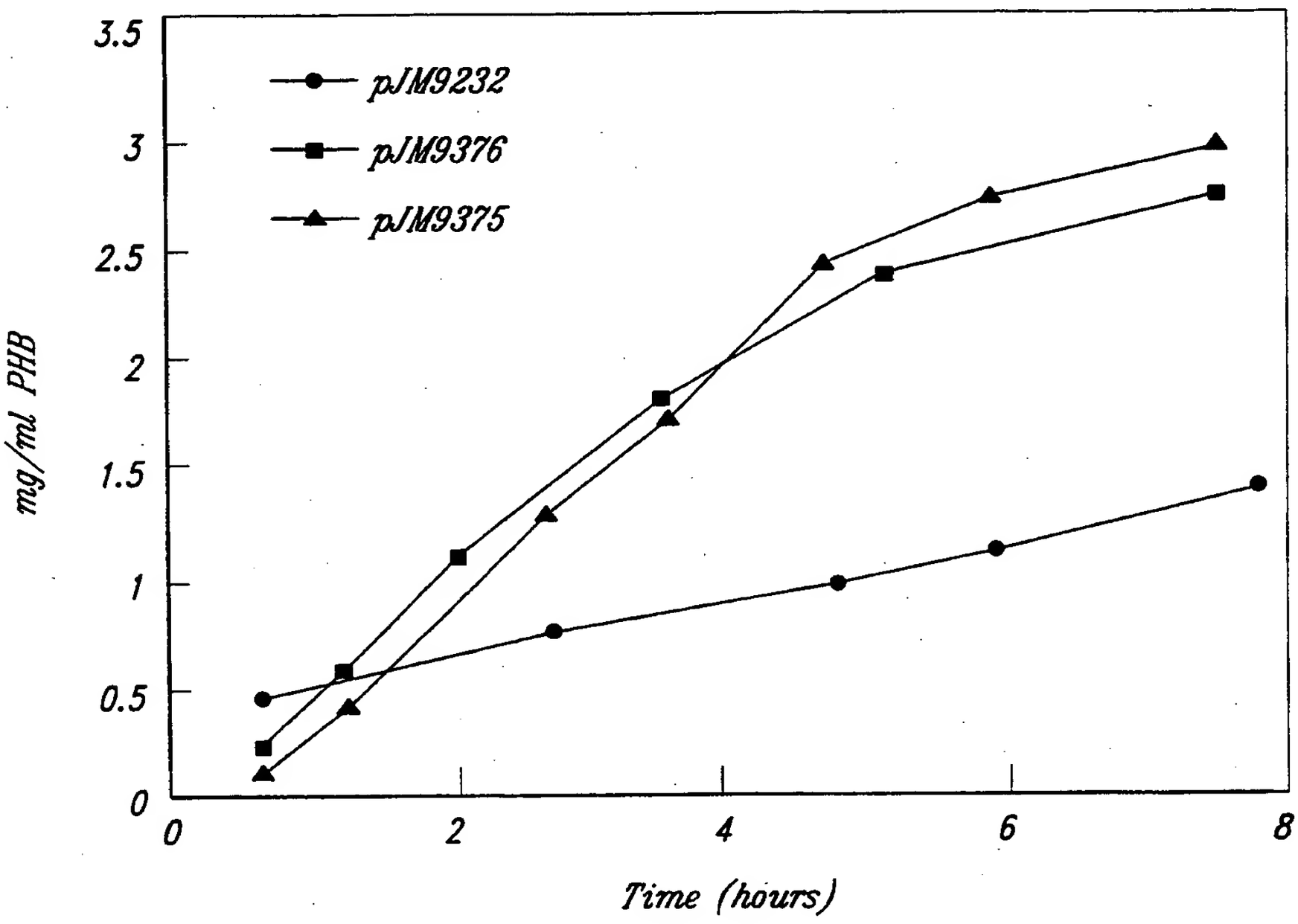

Comparison of PHB Symthesis in
Transcriptional and Translational Fusions
pJM9232
pJM9376
pJM9375
mg/ml PHB
Time (hours)
0
0.5
1
1.5
2
2.5
3
3.5
0
2
4
6
8

Fig. 19

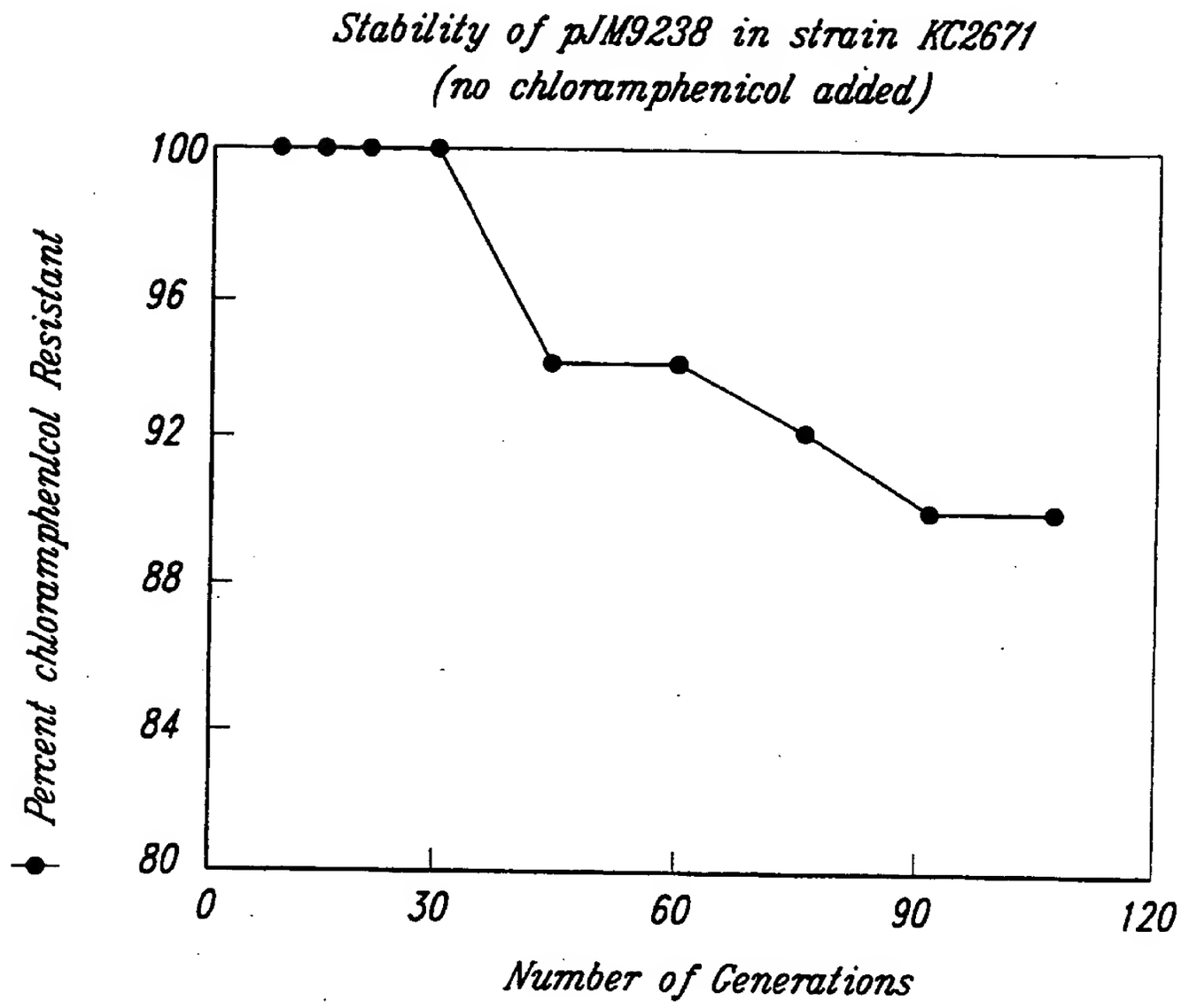


Fig. 20A

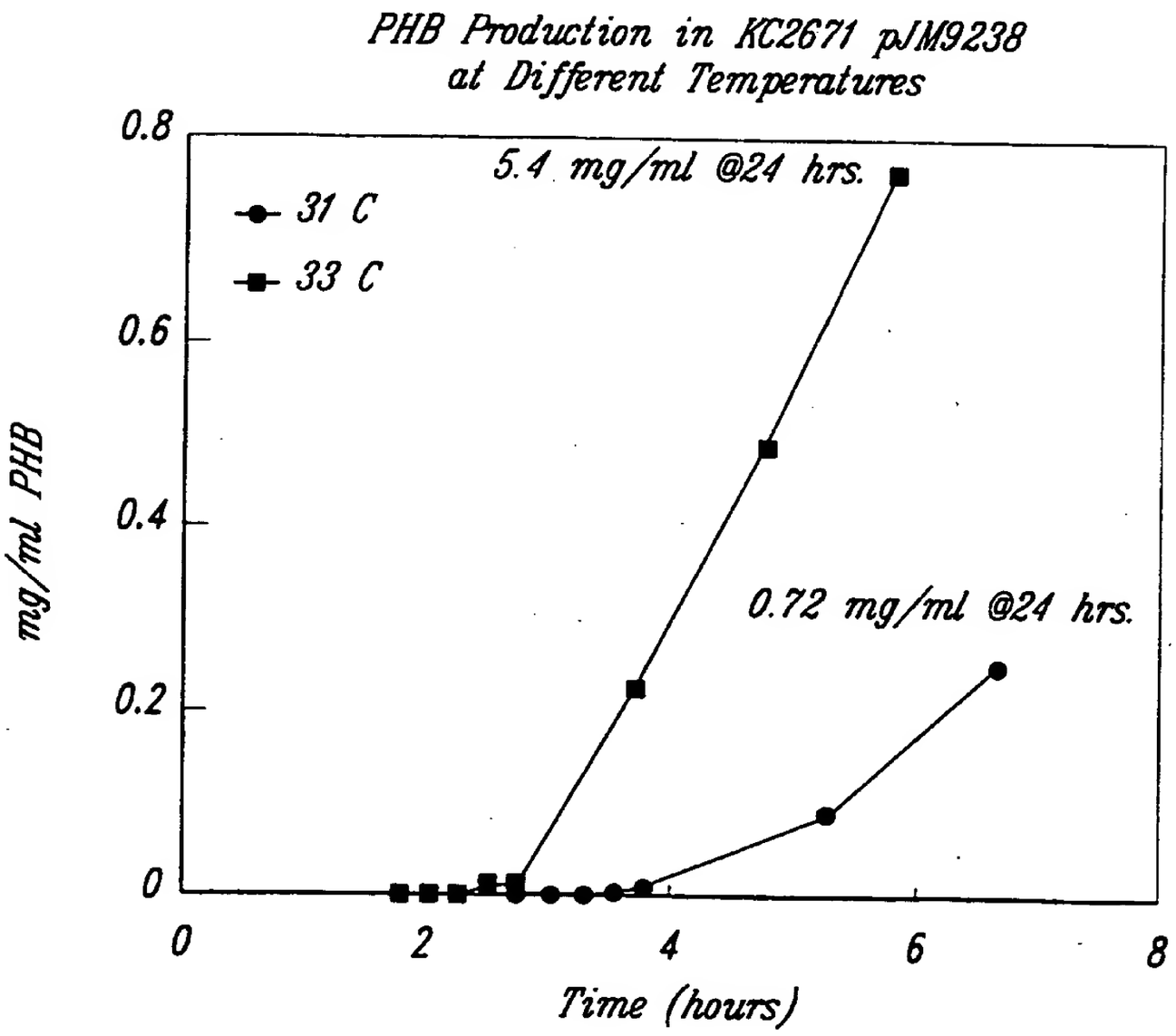


Fig. 20B

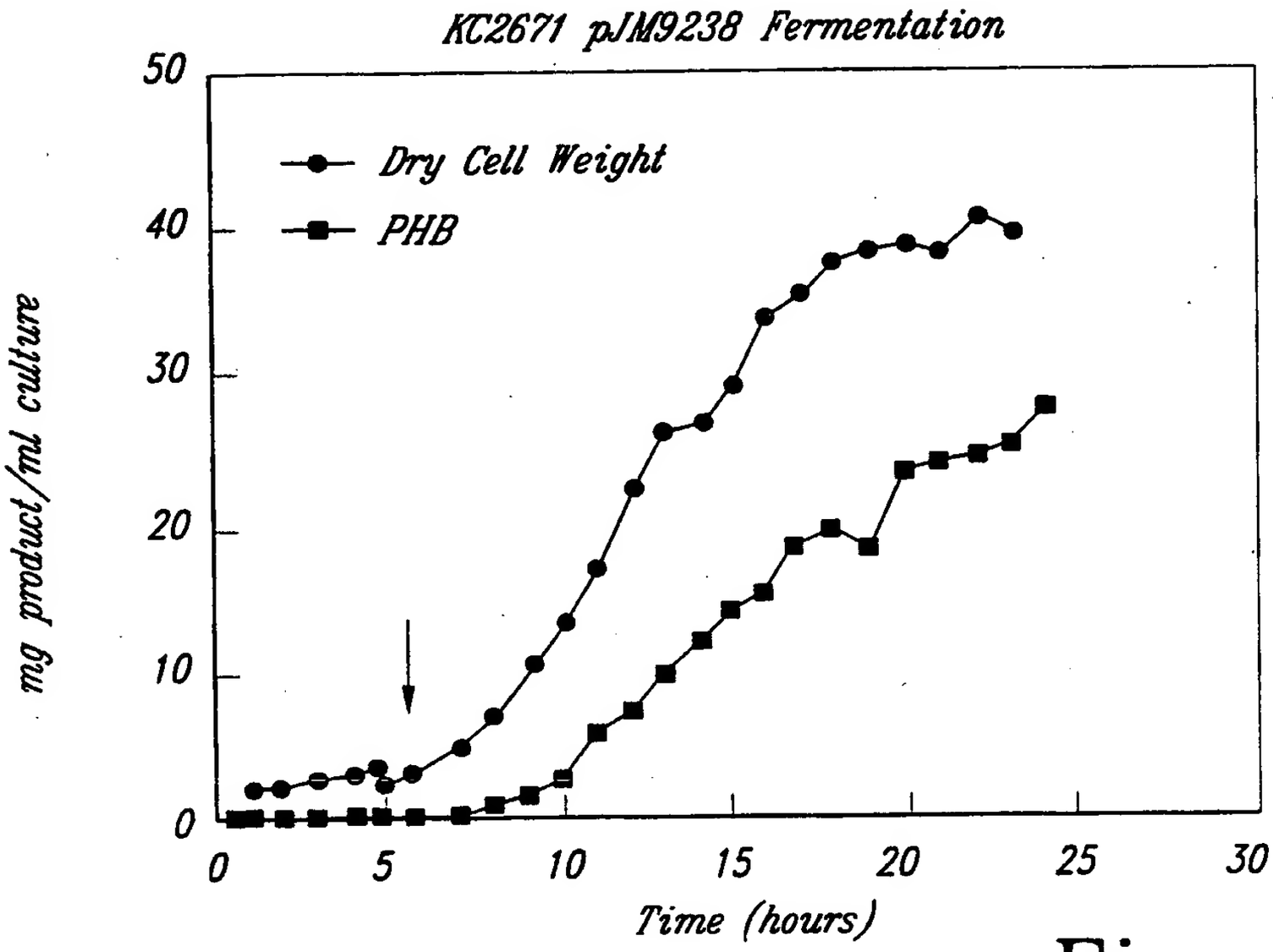


Fig. 21A

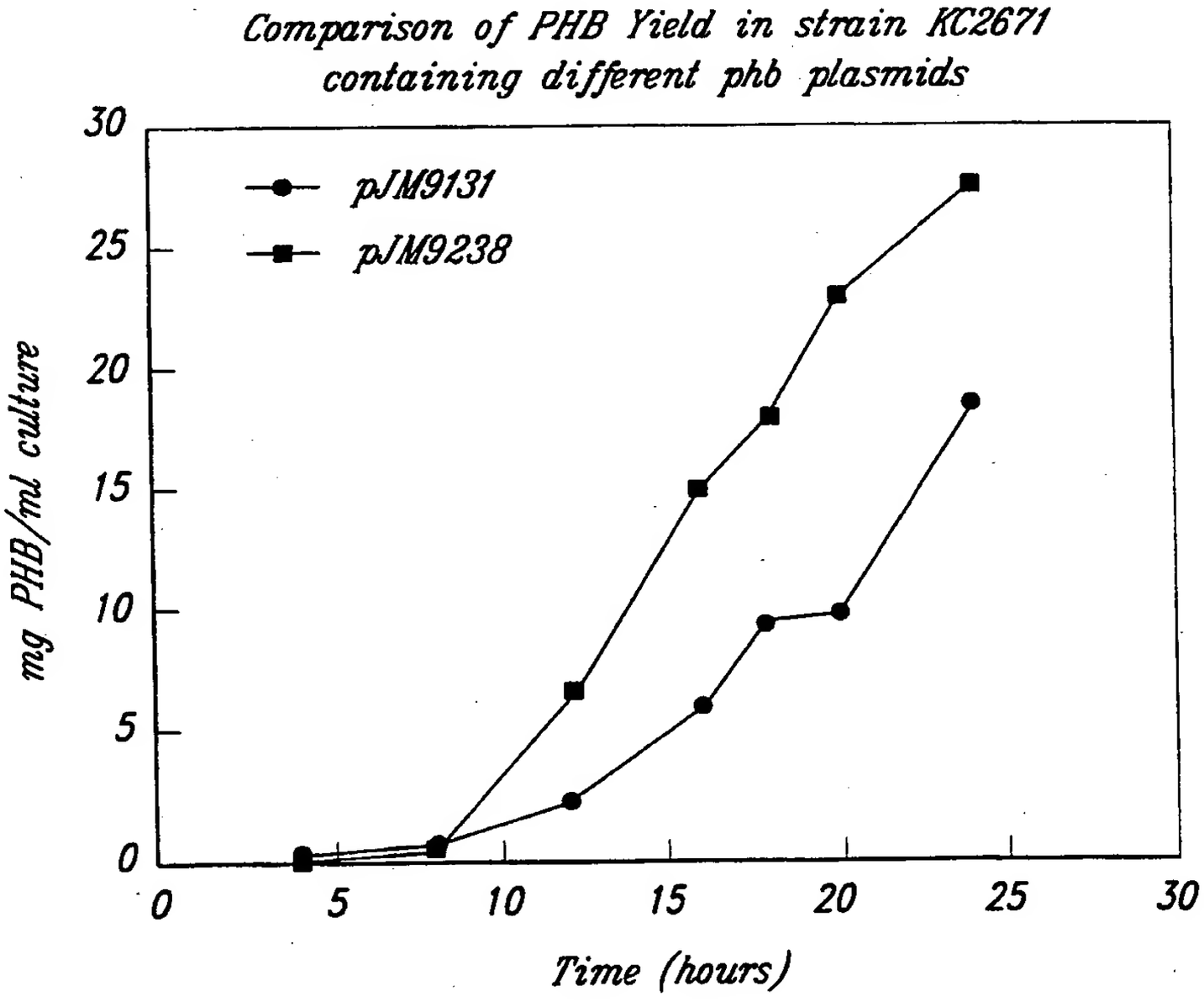


Fig. 21B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/01433

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12P7/62 C12N1/21 //(C12N1/21,C12R1:22), (C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 24633 (CENTER FOR INNOVATIVE TECHNOLOGY) 9 December 1993	23, 26-31, 35, 37-40, 48, 50-52,54
Y	see the whole document	1-22,24, 25,33, 34,36, 41,42, 44-47,53

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 June 1995	21. 06. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Van der Schaal, C

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,91 00917 (MASSACHUSETS INSTITUTE OF TECHNOLOGY) 24 January 1991 see the whole document especially page 16, second paragraph	1-22,24, 25, 29-34, 36, 39-42, 44-47, 51-53 54
Y	R. OLD AND S. PRIMROSE 'Principles of gene manupilation' 1989 , BLACKWELL SCIENTIFIC PUBLICATIONS , OXFORD see page 59, paragraph 3 - paragraph 5 see page 153, paragraph 3 - page 160, paragraph 1 see page 164, last paragraph	1-22,24, 25, 29-34, 36, 39-42, 44-47, 51-53
Y	J. SAMBROOK ET AL 'Molecular cloning' 1989 , COLD SPRING HARBOR LABORATORY PRESS see page 17.12 - page 17.13 see page 17.17 - page 17.27	1-22,24, 25, 29-34, 36, 39-42, 44-47, 51-53
Y	EP,A,0 136 829 (SUNTORY KABUSHIKI KAISHA) 10 April 1985 see the whole document	1-22,24, 25, 29-34, 36, 39-42, 44-47, 51-53
P,X Y	WO,A,94 21810 (CENTER FOR INNOVATIVE TECHNOLOGY) 29 September 1994 see the whole document	54 41,42,53

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/01433

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		EP-A-	0482077	29-04-92
		JP-T-	5500751	18-02-93
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		US-A-	5250430	05-10-93
EP-A-136829	10-04-85	JP-A-	60054685	29-03-85
		AU-B-	588220	14-09-89
		AU-A-	3259984	07-03-85
WO-A-9421810	29-09-94	NONE		